Territorial behaviour and testosterone in male black redstarts *(Phoenicurus ochruros)*: the ecophysiology of territoriality in a short distance migrant

Dissertation

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B.A. contributed to the design of the study, most of the field work, statistical analysis, and writing of the manuscript.

K.G.M. contributed to the design of the study (experiment song), fieldwork (song recordings) and the analysis of the song recordings.

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C.V. contributed to the analysis of the *in-situ* hybridization data.

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Chapter 2: Ignoring the challenge? Male black redstarts *(Phoenicurus ochruros)* do not increase testosterone levels during territorial conflicts but they do so in response to gonadotropin-releasing hormone.

B.A. contributed to the design of the study, field work, hormone analysis, statistical analysis, and writing of the manuscript.

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Chapter 3: Simulating winning in the wild — The behavioral and hormonal response of black redstarts to single and repeated territorial challenges of high and low intensity.

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Chapter 4: Testosterone affects song modulation during simulated territorial encounters in male black redstarts (*Phoenicurus ochruros*).

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Chapter 5: Testosterone does not drive territorial aggression, but emphasizes vocal behaviors in the territorial response in breeding black redstarts, *Phoenicurus ochruros.*

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Chapter 6: Autumnal territoriality and testosterone in a temperate zone songbird, the black redstart *(Phoenicurus ochruros)*.

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Summary

Natural plasma levels of testosterone in male temperate zone vertebrates vary markedly between individuals even when sampled during the same breeding stage. Social interactions between males are thought to be one major factor influencing short and long-term fluctuations in testosterone levels within individuals in most vertebrate classes. Furthermore, it is thought that territorial behaviour of temperate-zone vertebrates is regulated by testosterone, because seasonal testosterone profiles often closely match the occurrence of territorial behaviour in males. However, some species also defend territories outside the breeding season when testosterone levels are low. The degree to which testosterone facilitates territorial behaviour in these species is not well understood.

Black redstarts (*Phoenicurus ochruros*) are temperate zone songbirds that defend territories and sing during breeding when testosterone levels are elevated and during non-breeding when testosterone levels are low (chapter 1). High-altitude populations have a short breeding season with typically one brood, whereas low-altitude populations have a long breeding season and raise two or more broods. Thus, black redstarts are an ideal study species to explore (1) if and how tightly territorial behaviour is facilitated by testosterone across seasons (2) if testosterone levels are socially modulated and (3) if social modulation of androgen levels depends on the ecology of the population (high- versus low-altitude).

High- and low-altitude male black redstarts reacted aggressively to simulated territorial intrusions (STI), but they did not increase testosterone in response to this behavioural challenge. However, males of both populations would have had the physiological capacity to raise testosterone: males that did not show an increase in testosterone during STIs showed a pronounced increase in testosterone when injected with gonadotropin releasing hormone (GnRH). GnRH is released by the hypothalamus and via several steps stimulates the testes to produce testosterone. So far it was unknown, if species that show no androgen response to aggressive interactions have already maximally elevated testosterone levels. My data demonstrate that at least in black redstarts this is not the case. Furthermore, my data show that the length of the breeding season or the number of broods cannot universally explain between-species differences in androgen responsiveness to territorial challenges as males from the high- and the low-altitude population responded to simulated territorial intrusions and GnRH in a similar way (chapter 2).

The function of short-term increases in testosterone in other species is still barely understood. Testosterone may enhance persistence of aggression during a fight or it may facilitate the winner effect. California mice (*Peromyscus californicus*), for example, show a short-term increase in testosterone only after winning several territorial encounters. Studies testing this “winner-challenge”-hypothesis are, however, still rare and have been conducted under laboratory conditions only. However, in free-living black redstarts even repeated STIs did not result in an increase in androgens, although males enhanced their

behavioural response (chapter 3). Taken together, in this species, aggressive behaviour does not seem to influence plasma androgen levels. Thus, although the winner effect is a very general phenomenon among vertebrates, its regulation may differ between species and may be independent of sex steroids in some species.

However, territorial behaviour may still be facilitated by testosterone during breeding and during non-breeding. In both seasons brain areas involved in the production of song and neural areas associated with sexual arousal and aggression expressed receptors for androgens and oestrogens as well as the enzyme aromatase, which converts testosterone into oestradiol (chapter 1). In a further experiment, we therefore implanted male black redstarts during breeding and non-breeding with an androgen receptor blocker and an aromatase inhibitor to block both potential direct and indirect effects of testosterone on territorial behaviour. These experiments suggest that in black redstarts territorial behaviour as such may be decoupled from testosterone or its metabolites. Rather, testosterone or oestradiol seem to regulate components of the territorial repertoire that are specifically relevant in a breeding context and shift the emphasis of the territorial response to these components. In male black redstarts testosterone and oestradiol emphasize vocalizations relative to non-vocal behaviours in the territorial response (chapter 5). Furthermore, black redstarts increased their vocal performance in response to simulated territorial intrusions (chapters 4 and 6). This change in song structure seems to depend partly on testosterone or oestradiol, because both males that were implanted with the

blockers and males that were challenged during non-breeding did not show the full structural change of their song (chapter 4 and 6). Vocal performance is thought to be an honest indicator of male quality. My experiments suggest that testosterone and its metabolites may be the underlying mechanism.

In summary, the experiments demonstrate that androgen levels in male black redstarts are not socially modulated and interactions between males over territories cannot account for the high inter-individual variability in plasma testosterone levels between males. However, differences in testosterone levels may reflect male quality by influencing vocal performance during agonistic contexts. Further, the data show that in male black redstarts territorial behaviour per se is probably not facilitated by testosterone and its metabolites. Instead sex steroids seem to activate only one component of territorial behaviour during the breeding season, namely context-dependent changes in song structure. Our findings challenge the general believe of a strong link between testosterone and territorial behaviour. Furthermore, they suggest that species differ fundamentally to which degree territorial behaviour is regulated by testosterone or its metabolites on a seasonal basis. These differences may be directly related to variation in androgen responsiveness to male-male interactions.

Zusammenfassung

Wirbeltiermännchen der gemäßigten Zone unterscheiden sich stark in ihren Bluttitern für das Sexualhormon Testosteron; auch dann wenn alle Proben während des gleichen Brutstadiums entnommen wurden. Man geht davon aus, dass soziale Interaktionen zwischen Männchen zu Schwankungen im Testosteronspiegel führen. Außerdem vermutet man, dass das Territorialverhalten von Wirbeltieren durch Testosteron gesteuert wird, da Phasen ausgeprägten Territorialverhaltens oft mit erhöhten Testosterontitern einhergehen. Allerdings verteidigen einige Arten auch ausserhalb der Brutzeit ihre Territorien, wenn ihr Testosteronspiegel niedrig ist. Inwieweit Testosteron bei diesen Arten das Territorialverhalten steuert ist noch unklar.

Hausrotschwänze (*Phoenicurus ochruros*) sind Singvögel der gemäßigten Breiten, die sowohl während als auch ausserhalb der Brutzeit ein Territorium verteidigen (Kapitel 1). Hochgebirgspopulationen dieser Art sind auf eine sehr kurze Brutzeit beschränkt und können deshalb meist nur eine Brut großziehen. Populationen in niedriger gelegenen Lagen schaffen dagegen manchmal bis zu drei Bruten pro Saison. Hausrotschwänze sind daher eine ideale Modellart um den Zusammenhang zwischen Territorialverhalten und Testosteron zu erforschen. In meiner Doktorarbeit habe ich versucht folgende Fragen zu beantworten: Beeinflusst Testosteron das Territorialverhalten von Hausrotschwänzen sowohl während als auch ausserhalb der Brutzeit? Beeinflussen Revierkämpfe zwischen Männchen ihren Testosteronspiegel? Ist der Einfluß des Territorialverhaltens auf den Testosteronspiegel abhängig von

der Brutökologie der jeweiligen Population? Die Ergebnisse meiner Studien möchte ich im Folgenden kurz zusammenfassen.

Obwohl sowohl die Männchen der Hochgebirgs- als auch die Männchen der Flachlandpopulation aggressiv auf einen experimentellen Eindringling in ihr Territorium reagierten, kam es in beiden Fällen zu keiner erhöhten Hormonausschüttung. Physiologisch gesehen wären sie aber dazu in der Lage gewesen: nach Injektion des Neurohormons Gonadotropin-Releasing-Hormon (GnRH) hatten die Männchen deutlich höhere Testosteronspiegel als direkt nach der territorialen Interaktion. Normalerweise wird GnRH vom Hypothalamus (einer Gehirnregion) ausgeschüttet und führt über mehrere Zwischenschritte zur Produktion von Testosteron durch die Hoden. Bisher war unklar, ob bei Arten, die nach territorialen Auseinandersetzungen keinen Testosteronanstieg im Blut aufwiesen, der Testosteronspiegel bereits maximal erhöht war. Wie meine Daten zeigen, ist das zumindest bei Hausrotschwänzen nicht der Fall. Ferner legen meine Ergebnisse nahe, dass Unterschiede in der Brutökologie nicht generell Unterschiede in der Androgenantwort auf aggressive Interaktionen erklären können, da die getesteten Hochgebirgs- und Flachlandpopulationen hormonell sehr ähnlich auf einen experimentiellen Eindringling und GnRH reagierten (Kapitel 2).

Weshalb bei manchen Arten territoriale Auseinandersetzungen zu einem erhöhten Testosteronspiegel führen ist immer noch nicht vollständig geklärt. Es gibt Hinweise, dass Testosteron, die Ausdauer mit der ein Territorium verteidigt wird erhöht und den sogenannten “winner effect” steuert. Der „winner effect“

steht für eine höhere Wahrscheinlichkeit eine aggressive Interaktion zu gewinnen, wenn man bereits eine vorangegangene gewonnen hat. In Kalifornischen Mäuse (*Peromyscus californicus*), zum Beispiel, ist der Testosteronspiegel erst nach mehreren gewonnen Revierkämpfen erhöht. Bisher gibt es allerdings nur sehr wenige Studien, die einen Zusammenhang zwischen dem “winner effect” und Testosteron gezeigt haben. Zudem wurden alle bisherigen Studien im Labor durchgeführt. Die von mir untersuchten freilebenden Hausrotschwänze erhöhten ihren Testosteronspiegel auch nach mehrfacher Konfrontation mit einem experimentellen Eindringling in ihr Territorium nicht, obwohl sie ihre Verhaltensantwort deutlich verstärkten (Kapitel 3). Aggressives Territorialverhalten hat bei dieser Art also keinen Einfluß auf den Testosteronspiegel. Auch wenn der „winner effect“ unter Wirbeltieren sehr weit verbreitet ist, scheint seine Steuerung zwischen verschiedenen Arten stark zu variieren und ist bei manchen Arten wahrscheinlich unabhängig von Testosteron. Diese Befunden schließen dennoch eine Testosteron-abhängige Steuerung des Territorialverhaltens nicht aus. Sowohl während als auch außerhalb der Brutzeit finden sich Rezeptoren für Testosteron und Östrogen sowie das Enzym Aromatase (wandelt Testosteron in Östrogen um) in Gehirngebieten, die für die Produktion von Gesang und für Sexual- und Aggressionverhalten entscheidend sind (Kapitel 1). In einem weiteren Versuch habe ich deshalb bei Hausrotschwanzmännchen in beiden Jahresabschnitten die Bindung von Testosteron an dessen Rezeptoren im Gehirn und dessen Umwandlung zu Östrogen inhibiert. Diese Versuche legen nahe, dass bei Hausrotschwänzen

Territorialverhalten an sich nicht an Testosteron oder seine Umwandlungsprodukte gekoppelt ist. Vielmehr scheint Testosteron nur jene Elemente des Territorialverhalten zu regulieren und hervorzuheben, die besonders während der Brutzeit von Bedeutung sind. Bei Hausrotschwänzen verstärken Testosteron oder seine Umwandlungsprodukte Vokalisationen gegenüber nicht-vokalen Verhaltensweisen (Kapitel 5). Hausrotschwänze erhöhten ihre Gesangsleistung als Antwort auf einen experimentellen Eindringling in ihr Territorium (Kapitel 4 und 6). Diese Veränderung in der Struktur ihres Gesangs scheint auf der Wirkung von Testosteron zu beruhen: Männchen, bei denen die Bindung von Testosteron an seine Rezeptoren blockiert wurde sowie Männchen während der Nichtbrutzeit im Herbst, wenn ihr Testosteronspiegel natürlicherweise niedrig ist, veränderten ihren Gesang nur teilweise als Antwort auf einen experimentellen Eindringling in ihr Territorium (Kapitel 4 und 6). Die Gesangsleistung eines Männchens gibt vermutlich Aufschluss über seine Qualität als Reproduktionspartner. Testosteron ist also wahrscheinlich der zugrundeliegende Mechanismus, der die Qualität eines Männchens in ein „ehrliches“ Gesangssignal übersetzt.

Abschliessend lässt sich sagen, dass der Testosteronspiegel von Hausrotschwanzmännchen nicht durch Interaktionen mit anderen Männchen beeinflusst wird. Territoriale Auseinandersetzungen können daher auch nicht erklären weshalb sich Hausrotschwanzmännchen so stark in ihren Testosterontitern unterscheiden. Wahrscheinlicher spiegelt diese Varianz aber unterschiedliche Qualitäten der Männchen wieder, die in den unterschiedlichen

Gesangsleistungen bei territorialen Auseinandersetzungen zum Ausdruck kommen. Außerdem läßt sich aus meinen Daten folgern, dass bei Hausrotschwänzen nicht die gesamte Territorialantwort von Testosteron und seinen Umwandlungsprodukten gesteuert wird, sondern nur eine Komponente davon, nämlich Kontext – abhängige Veränderungen in der Gesangsstruktur. Damit stellen meine Ergebnisse den generell angenommenen starken Zusammenhang zwischen Territorialverhalten und Testosteron in Frage. Es scheint jedoch fundamentale Unterschiede zwischen den Arten zu geben inwieweit Territorialverhalten von Testosteron gesteuert wird und davon scheint auch abzuhängen, ob Interaktionen zwischen Männchen deren Testosteronspiegel beeinflussen oder nicht.

General introduction

The role of sex steroids in a seasonally changing environment

Most longer lived animal species - regardless whether they breed in the arctic, the temperate-zones or in the tropics - have to cope with a seasonally changing environment. Therefore, in most of these species energetically costly life-history stages are restricted to times when weather conditions are favourable. Northern temperate songbirds, for example, typically breed from spring to early summer and moult their feathers in late summer when food is still plenty. To avoid the winter period many songbirds migrate to southern regions with more benign weather and food conditions. In response to these seasonal changes animals, therefore, go through different life-history stages that are accompanied by changes in behaviour and physiology and allow them to maximize their reproductive success in a largely predictable changing environment. For example, sexual behaviours are most advantageous during the breeding season. Therefore, the physiology and behaviour of individuals need to be matched with environmental conditions and social context. This is the major role of sex steroids like testosterone. Testosterone integrates environmental and social cues to prepare male vertebrates for reproduction: it stimulates the production of sperm by the testes, promotes the expression of secondary sex characteristics and facilitates the expression of relevant behaviours like territoriality, courtship and copulation (Adkins and Pniewski 1978; Nelson 2005). Thus, testosterone induces the simultaneous expression of different traits that are relevant for breeding. Accordingly, in many male vertebrates testosterone levels are elevated during

the breeding season and are low during non-breeding. At the beginning of the breeding season, the increasing day length stimulates gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus to secrete GnRH into the pituitary (Dawson, King et al. 2001). The pituitary in turn releases luteinizing hormone (LH) into the blood stream and stimulates the regressed testes to grow and produce testosterone. As testosterone is distributed by the blood stream it can exert its effects on virtually every organ provided its cells express receptors for this hormone. Sex steroids are small lipophilic molecules and can, therefore, pass through the membrane of cells, bind to receptors inside the cell and are then transported into the nucleus where they can activate the transcription of respective genes (Nelson 2005). Many effects of testosterone on behaviour are not accomplished by testosterone itself, but by its conversion product oestradiol. Testosterone is then locally aromatized into oestrogens in brain areas that have been shown to be especially relevant for sexual and aggressive behaviours (Schlinger and Callard 1990). It is now known that some cells also express membrane-bound receptors for sex steroids. These activate signal transduction pathways and lead to effects of testosterone on behaviour that are faster than those achieved by the classic activation of genes (Remage-Healey and Bass 2006).

Territorial behaviour and testosterone

Territorial behaviour and testosterone during the breeding season

Males of many vertebrate species are highly territorial or aggressive against other males during the breeding season when they compete for access to fertile females. During the non-breeding season, these males often readily accept the proximity of conspecific males (e.g. Feare 1984, Lincoln, Guinness et al. 1972). Thus, besides seasonal changes in sexual behaviours, seasonal changes in territorial behaviour and aggression occur in many vertebrates. Accordingly, it has been shown in a variety of species that territorial behaviour is facilitated by testosterone (Lincoln, Guinness et al. 1972; Wingfield, Moore et al. 2006). The role of sex steroids like androgens and oestrogens in the activation of sexual behaviours is fairly established and seems to be pretty conserved across vertebrates (Adkins and Pniewski 1978; Adkins-Regan 1981; Nelson 2005; Fusani 2008). In contrast, - and despite many years of research - the relationship between testosterone and territorial behaviour and aggression is far less clear and seems to be more subject to variation between species (Wingfield, Moore et al. 2006). There may be several reasons for this. First, aggression can be expressed in different contexts and situations. It may, therefore, serve different purposes that require the expression of distinct agonistic behaviours. Further, aggression may be intra- and intersexual, it may be over food, territories and mating partners and it may be directed against conspecifics or against individuals of other species, e.g. predators (Wingfield, Moore et al. 2006). Focussing on

territorial behaviour as a special case of aggression reveals that even then differences in context may be present. For example, territorial behaviour in songbirds is expressed most prominently during the breeding season. During breeding males compete over territories and access to mates. In species that only defend a territory during the breeding season a close correlation between the intensity of territorial behaviour and testosterone is found (Wingfield, Hegner et al. 1990). However, some songbirds even defend territories outside the breeding season when testosterone levels are low (Schwabl and Kriner 1991; Wingfield 1994; Hau and Beebe 2011; Marasco, Fusani et al. 2011).

During the breeding season testosterone is thought to shift the time and energy budget of an individual from self-maintenance and long-term survival towards mating effort and reproduction (Hau 2007). Accordingly, it has been shown, that in some species males implanted with testosterone during the breeding season increase the size of their territories (Chandler, Ketterson et al. 1994), territorial behaviour (Wingfield 1984) and song rate (Hau, Wikelski et al. 2000; Van Duyse, Pinxten et al. 2002; Sartor, Balthazart et al. 2005). Consequently, prolonged high levels of testosterone may also entail behavioural and physiological costs (Marler and Moore 1988; Dufty 1989; Wingfield, Lynn et al. 2001). However, individual correlations between testosterone and territorial behaviour are rarely found (Johnsen 1998; McGlothlin, Jawor et al. 2007). Furthermore, testosterone levels do not only vary on a seasonal basis, but also within the breeding season and depending on the mating system (Wingfield, Hegner et al. 1990; Hirschenhauser and Oliveira 2006). For example, in socially

monogamous species with biparental care testosterone levels are highest (and show the largest differences between individuals) at the very beginning of the breeding season, when conflicts between males over territories and mates are most intense (Wingfield, Hegner et al. 1990). Thus, because of experimental and correlational evidence and the potential costs of high prolonged levels of testosterone, it has been suggested that individuals only increase testosterone levels above breeding baseline levels when necessary (Wingfield, Hegner et al. 1990) - that is during periods of social instability when dominance hierarchies or territories have to be established (Ramenofsky 1984). These ideas are the basis of the “challenge hypothesis” (Wingfield, Hegner et al. 1990). Evidence for this hypothesis came from studies on temperate-zone songbirds. For example, song sparrows show a rise in testosterone levels above breeding season baseline levels when they are challenged with a simulated territorial intrusion (STI) experiment creating a situation of social instability (Wingfield 1985; Wingfield and Wada 1989). The challenge hypothesis found broad resonance and has been tested in a variety of vertebrate species. Most of these studies confirm the seasonal predictions of the challenge hypothesis that depending on the mating system high levels of testosterone should correlate with times when interactions between males are most intense during the breeding season (Hirschenhauser, Winkler et al. 2003; Hirschenhauser, Taborsky et al. 2004; Hirschenhauser and Oliveira 2006). Socially monogamous species with biparental care should only show a peak in testosterone levels at the beginning of the breeding season. In contrast, individuals of polygynous species that compete for females during the

whole breeding season and do not provide paternal care should have elevated testosterone levels during the entire breeding season. However, the assumption that variations in testosterone levels above breeding season levels are *caused* by aggressive interactions between males was only confirmed in some species, many others do not behave in the way predicted by the challenge hypothesis (for a review see Goymann, Landys et al. 2007). So far three ecological hypotheses, the essential paternal care, the short breeding season and the number of broods hypothesis, have been put forward to explain between-species differences in the androgen responsiveness to male-male interactions (see Goymann 2009). These hypotheses are not mutually exclusive and are based on the general idea that biparental species living in environments with strong time or resource limits for breeding should not increase testosterone during territorial intrusions, because high levels of testosterone may interfere with paternal care (Hunt, Hahn et al. 1999; Goymann, Landys et al. 2007; Landys, Goymann et al. 2007; Lynn 2008; Goymann 2009).

Furthermore, the challenge hypothesis goes beyond the classical idea of hormones facilitating behaviour: behaviour can also feedback on hormone levels (Harding and Follett 1979; Adkins-Regan 2005). Although the influence of behaviour on hormones is now well accepted and found in all vertebrate species from humans to fish (e.g. Hirschenhauser, Taborsky et al. 2004; Archer 2006), the actual function of short-term increases of sex steroids in response to sexual activity or territorial fights is still unclear. It has been suggested that testosterone may increase aggression during an on-going fight, ensures the persistence of

aggression after a fight and mediates the winner effect (Wingfield 1994; Oliveira, Silva et al. 2009).

Territorial behaviour and testosterone across seasons

As mentioned above, in some species the correlation between testosterone and territorial behaviour is disrupted as they also defend territories in the non-breeding season when testosterone levels are low (Schwabl and Kriner 1991; Wingfield 1994; Canoine and Gwinner 2002). Only few studies so far have investigated the relationship between testosterone and territorial behaviour in species that also defend territories in a non-reproductive context and these studies have yielded conflicting results. There is good evidence that in song sparrows *(Melospiza melodia morphna)* sex steroids facilitate territorial behaviour in the non-breeding season although androgens are undetectable in the blood plasma (Soma, Sullivan et al. 1999). In that case territorial behaviour seems to be regulated by local production of androgens and oestrogens in relevant brain areas (Soma, Sullivan et al. 2000; Soma, Tramontin et al. 2000; Soma, Schlinger et al. 2003) derived from steroid precursor molecules of non-gonadal origin (Soma and Wingfield 2001). However, studies on other songbirds suggest that in these species testosterone only plays a role in the regulation of territorial behaviour during the breeding season (Schwabl and Kriner 1991; Canoine and Gwinner 2002; Hau and Beebe 2011; Marasco, Fusani et al. 2011). Furthermore, although testosterone correlates with territorial behaviour during the breeding season, experimental evidence actually suggests that territorial behaviour as a

whole is not facilitated by testosterone in most species even during the breeding season (see chapter 5, Table 1). Similar to courtship, territorial behaviour comprises a whole suite of different components that may be regulated by different hormonal mechanisms (Fusani, Gahr et al. 2001; Van Duyse, Pinxten et al. 2002; Fusani 2008; Sperry, Wacker et al. 2010).

One major component of territorial behaviour is song which is often studied separately from non-vocal territorial behaviours. Studies on song behaviour indicate that although several species sing also outside the breeding season, nonbreeding song may be quantitatively and/or qualitatively different from breeding season song. For example, some species produce more repetitive elements (DeWolfe, Kaska et al. 1974; Smith, Brenowitz et al. 1997; Leitner, Voigt et al. 2001; Voigt and Leitner 2008), longer songs (Riters, Eens et al. 2000) and more stereotyped song (Smith, Brenowitz et al. 1997) in spring than in autumn. In some species these changes are correlated with HVC size (Smith, Brenowitz et al. 1997), but in others not (Fusani, Van't Hof et al. 2000; Smulders, Lisi et al. 2006). HVC is a brain nucleus and part of the song control system in songbirds. It is androgen sensitive (Gahr and Metzdorf 1997) and thought to control motor output during singing (Brenowitz, Margoliash et al. 1997). It is argued that a larger HVC during spring is related to a larger song repertoire, a higher song rate and that it facilitates the production of more complex song (Brenowitz 1997; Smith, Brenowitz et al. 1997). This view is currently debated though, as some of the reported seasonal changes in HVC size may depend on the delineation method used (Gahr 1997). However, there is evidence that

androgen and oestrogen sensitivity within the HVC changes seasonally (Gahr and Metzdorf 1997; Soma, Hartman et al. 1999). Furthermore, testosterone may facilitate the production of song in a reproductive context by activation of song areas outside the song control system. Especially aromatization of testosterone to oestrogens in the pre-optic area seems to be important in this respect (Foidart, Silverin et al. 1998; Riters, Eens et al. 2000; Soma, Schlinger et al. 2003).

Thus, although it is accepted that testosterone plays a role in the organization and activation of song (Bolhuis and Gahr 2006) and territorial behaviour in general, it is still unclear to what extent testosterone facilitates these behaviours in species that sing and defend territories also outside the breeding season.

Aims of the study

Although it is clear that there is a general relationship between testosterone and territorial aggression, the extent to which testosterone plays a role is not well understood (Wingfield, Hegner et al. 1990; Adkins-Regan 2005; Nelson 2005; Wingfield 2005; Wingfield, Moore et al. 2006). We therefore aimed at elucidating the role of testosterone in regulating territorial behaviour across seasons and if territorial behaviour can explain variation in testosterone levels between individuals during breeding in a temperate-zone songbird. We studied the interrelationship between testosterone and territorial behaviour at all levels by collecting correlational and experimental data and by including all components of territorial behaviour from overt aggression, approach behaviour and song (song

rate and song structure). Thus, although this work was restricted to one particular species, the black redstart (*Phoenicurus ochruros*, Fig. 1), we are confident that our results apply to a broader range of songbirds and vertebrates with similar life histories.

Figure 1. Adult male black redstart bringing food to its nestlings.

The black redstart has a largely predictable and well known life-history cycle (Menzel 1995; Landmann 1996) and is a highly territorial, socially monogamous songbird with bi-parental care. It covers a large breeding range from high to low altitudes within Europe (Landmann 1996; Weggler 2006, Fig. 2). High-altitude populations have a short breeding season with typically one brood, whereas low-altitude populations have a long breeding season and raise two or more broods (Landmann 1996). Furthermore, black redstarts show a resurgence of territorial behaviour in autumn after moult before they leave their breeding grounds (Weggler 2000; Nicolai 2005).

Figure 2. Typical breeding habitats of black redstarts. Left side: breeding habitat of the low-altitude population in Bavaria. Right side: breeding habitat of the high-altitude population in Southern Tyrol.

In **chapter 1** we assessed the correlational basis between territorial behaviour and testosterone in male black redstarts collecting data from March, when they arrive on their breeding grounds until October when they leave for their wintering grounds. We collected behavioural data in different contexts (spontaneous, in response to a simulated territorial intrusion) and physiological data (circulating sex steroid levels, sex steroid receptor expression in relevant brain areas). If there is a tight link between territorial behaviour and testosterone, the response of males to a simulated territorial intrusion should be strongest at the beginning of the breeding season when testosterone levels are elevated and

lowest during the non-breeding season in autumn when testosterone levels are presumably very low. Similarly, song output should be highest at the beginning of the breeding season and lowest during non-breeding in autumn. Furthermore, if these seasonal differences in behaviour are driven by the effects of testosterone in the relevant brain areas, androgen sensitivity or sensitivity for its metabolites should be lower in these brain areas and the song control nucleus HVC should be smaller in autumn than in spring.

In **chapters 2 and 3** we explored factors that may explain variation within and between species in their androgen responsiveness to simulated territorial intrusions ranging from life history differences to physiological and social factors.

In **chapter 2** we tested the short breeding season / number of broods hypothesis by comparing two populations of black redstarts that breed at different altitudes and therefore have different life histories: a high altitude population with typically one brood per breeding season and a low altitude population with up to three broods per breeding season. Following the predictions of the two hypotheses we expected that the population with a short breeding season would not show an increase in testosterone during simulated territorial intrusions to avoid the negative effects of testosterone on male parental care. The population with the long breeding season, in contrast, should show an increase in testosterone as the value of a brood is not as high as in the single-brooded population and, therefore, the boost in territorial behaviour gained by the increase in testosterone may outweigh its negative effects. This was the first study to test these hypotheses in populations of the same species. Furthermore,

we tested if differences in hypothalamic-pituitary-gonadal axis physiology may explain differences in androgen responsiveness between species: species that lack an increase in androgen levels in response to simulated territorial intrusions may already have maximally elevated androgen levels. Male black redstarts were caught in a control situation or during a simulated territorial intrusion and injected with GnRH to stimulate the testes to produce a maximum amount of testosterone. This idea had not yet been tested so far.

In **chapter 3** we tested three hypotheses that may explain the lack of a hormonal response of various species to single simulated territorial intrusions: (1) Multiple (winning) territorial encounters may be needed to increase testosterone (Oyegbile and Marler 2005) or (2) simulated territorial intrusions may create a losing experience for territory holders, during which territorial birds do not increase testosterone (Kempenaers, Peters et al. 2008). Finally, (3) the hormonal response may depend on intrusion intensity. We evaluated these hypotheses based on patterns of aggressive behavior that are indicative of winning and losing in other species (e.g. (Hsu, Earley et al. 2006)) and differences in androgens and corticosterone to single versus repeated simulated territorial intrusions with low- or high-level intensity encounters. If male black redstarts should require multiple territorial interactions before they show a rise in testosterone, we predicted that redstarts caught after experiencing and "winning" three consecutive simulated territorial intrusions before capture should express higher levels of testosterone than individuals caught after just one simulated territorial intrusion. With respect to behavior, challenged territorial males should

intensify their behavioural response over days and should react stronger towards the more threatening high-level intruders than towards the less threatening low-level intruders.

The experiments presented in **chapters 2 and 3** also aimed at answering if testosterone levels are highest and most variable at the beginning of the breeding season due to interactions between males; hence, behaviour may feed back on hormone levels.

In **chapters 4 and 5** we assessed the effect of testosterone on territorial behaviour (including song rate and song structure) during the breeding season by blocking the action of testosterone on receptors in the brain and its conversion to oestrogens. We hypothesized that testosterone may not change territorial behaviour per se, but only some aspects of territoriality that are especially relevant in a reproductive context, e.g. that may be attractive to females.

In **chapter 4** we studied a particular component of the territorial response, namely song. We asked if male black redstarts change their song (song output and structure) in response to a simulated territorial intrusion and if this is dependent on testosterone and/or its metabolite oestradiol. If song in black redstarts is regulated by testosterone, blocker-treated males should show a reduced vocal response compared to control males.

In **chapter 5** we compared the whole repertoire of territorial behaviours in response to simulated territorial intrusions of control males with that of blocker implanted males. If testosterone is playing a key role in the resource allocation

for competitive behaviour, we would expect blocker-treated males to invest less in those behaviours than control males.

In **chapter 6** we tested if territorial behaviour in different seasons and thus in different contexts (reproductive or not) is regulated by the same endocrine mechanism as during the breeding season. To assess the role of sex steroids in the regulation of non-breeding territorial behaviour we compared behavioural responses towards a simulated territorial intrusion in spring and autumn (see chapters 4 and 5 for methods). If territorial behaviour is facilitated by testosterone also in autumn when testosterone levels are low, blocker treated males should respond less to a simulated territorial intrusion and show a weaker vocal response than control males. If testosterone does not regulate autumn territoriality in black redstarts, the two treatment groups should not behave significantly different in response to a simulated territorial intrusion.

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Chapter One

Seasonality, hormones, brain and behaviour in male black redstarts, *Phoenicurus ochruros*

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Abstract

It is thought that territorial behaviour is regulated by testosterone in male vertebrates because in many species seasonal peaks in testosterone closely match with periods of intense competition between males for territories and mating partners. However, in some species territorial behaviour is also expressed outside a breeding context and the degree to which testosterone facilitates territorial behaviour in these species is not well understood. We, therefore, studied territorial behaviour and its hormonal basis in male black redstarts – a temperate-zone songbird that defends territories during breeding and in autumn after feather moult and just before migrating to its wintering grounds. We compared the response to simulated territorial intrusions between breeding and non-breeding. Furthermore, we assessed seasonal differences in song output and structure. To determine if testosterone influences song structure we implanted males with an anti-androgen and an aromatase inhibitor to block

both direct and indirect effects of testosterone. In addition, we determined the expression patterns of androgen- and oestrogen receptor as well as aromatase mRNA in the forebrain song control nuclei and in the diencephalon during breeding and non-breeding. The results suggest that the relationship between testosterone and territorial behaviour in male black redstarts is complex: testosterone does not seem to regulate non-vocal territorial behaviours, but the hormone may be involved in the regulation of song. Testosterone may influence song output and it may stimulate males to produce more repetitive elements in their song during breeding compared to non-breeding. However, in male black redstarts the seasonal decrease in repetitive elements does not seem to depend on testosterone or its conversion to oestrogens: treatment with blockers had no significant effect on the number of elements produced. The seasonal changes in the role of song in the territorial response of black redstarts may be regulated by conversion of testosterone to oestradiol in the pre-optic area.

Introduction

Most animals face seasonal environments that vary in environmental conditions such as temperature and precipitation and the availability of resources such as food and shelter. In the temperate zones, for example, winter is associated with lower temperatures and a sharp decline in plant productivity, resulting in low availability of food. To persist in such environments and maximize reproductive success animals need to cope with these largely predictable changes and time

their life-cycle accordingly. Different stages of the life-cycle require distinct physiological and behavioural adaptations which are often regulated by hormones. Especially the sex-steroid testosterone facilitates physiological requirements and behaviours that are important for breeding in male vertebrates: this hormone is required for spermatogenesis, the development of secondary sex characteristics and it facilitates sexual and territorial behaviours (Adkins-Regan 2005; Nelson 2005). Accordingly, seasonal testosterone profiles often correlate with sexual and territorial behaviours (Wingfield, Hegner et al. 1990) and testosterone levels are highest at the beginning of the breeding season when interactions among males are most intense (Dawson 1983; Silverin, Viebke et al. 1986; Ball and Wingfield 1987; Morton, Peterson et al. 1990; Silverin 1993; Van Duyse, Pinxten et al. 2003). Competition for territories and mating partners may increase circulating levels of testosterone (Wingfield, Hegner et al. 1990), but see (Goymann, Landys et al. 2007; Goymann 2009; Apfelbeck and Goymann 2011). In general, testosterone levels then decline to breeding baseline levels, but in some species peak once more when females are fertile (Goymann and Landys, J Avian Biol, in press; Wingfield 1984). While testosterone orchestrates these physiological and behavioural requirements for breeding in males, prolonged elevated levels of this hormone may have costs (Dufty 1989; Wingfield, Lynn et al. 2001; Roberts, Buchanan et al. 2004). For example, testosterone shifts the time budget of males towards activities associated with territory defence and mating away from parental duties (Wingfield, Moore et al. 2006; McGlothlin, Jawor et al. 2007). Thus, periods with high levels of testosterone should be

restricted to times when they are most advantageous, such as at the beginning of the breeding season and should be avoided during times when offspring requires parental care.

However, the correlation between behaviours expressed in a reproductive context and testosterone is not always that clear: many songbird species also defend territories outside the breeding season when testosterone levels are low (Schwabl and Kriner 1991; Wingfield 1994; Canoine and Gwinner 2002; Landys, Goymann et al. 2010; Apfelbeck and Goymann 2011; Hau and Beebe 2011). However, territoriality may still be facilitated by testosterone in these species. For example, blocking the aromatization of testosterone to oestrogens, leads to a reduced response to a simulated territorial intruder in song sparrows (*Melospiza melodia morphna*, (Soma, Tramontin et al. 2000) although testes are regressed and circulating testosterone is low. In such instances, testosterone may be produced either directly in the brain or it may be derived from non-gonadal sources, e.g. dehydroepiandrosterone (DHEA) that is produced by the adrenals (Soma and Wingfield 2001). Alternatively, brain sensitivity for low levels of sex steroids may be increased during the non-breeding season (Canoine, Fusani et al. 2007). Yet in the few other species studied so far, there is no indication that testosterone facilitates territorial behaviour also during the non-breeding season (Schwabl and Kriner 1991; Canoine and Gwinner 2002; Hau and Beebe 2011; Marasco, Fusani et al. 2011).

One major component of territorial behaviour is song, which is often studied separately from non-vocal territorial behaviours. Studies on song

behaviour indicate that although several species sing also outside the breeding season, non-breeding song may differ in quantity and quality from breeding season song. For example, some species produce more repetitive elements (DeWolfe, Kaska et al. 1974; Smith, Brenowitz et al. 1997; Leitner, Voigt et al. 2001; Voigt and Leitner 2008), longer songs (Riters, Eens et al. 2000) and more stereotyped song (Smith, Brenowitz et al. 1997) in spring than in autumn.

In some species these changes are correlated with the size of the HVC, which is a brain nucleus and part of the song control system in songbirds (Smith, Brenowitz et al. 1997). But in other species changes in song are not related to HVC size (Fusani, Van't Hof et al. 2000; Smulders, Lisi et al. 2006). The HVC is androgen sensitive (Gahr and Metzdorf 1997) and thought to control motor output during singing (Brenowitz, Margoliash et al. 1997). It has been argued that a larger HVC during spring is related to a larger song repertoire, a higher song rate and that it facilitates the production of more complex song (Brenowitz 1997; Smith, Brenowitz et al. 1997). But this view is debated because some of the reported seasonal changes in HVC size may depend on the delineation method used (Gahr 1997). There is evidence that androgen and oestrogen sensitivity within the HVC changes seasonally (Gahr and Metzdorf 1997; Soma, Hartman et al. 1999). Furthermore, testosterone may facilitate the production of song in a reproductive context by activation of song areas outside the song control system. Especially aromatization of testosterone to oestrogens in the pre-optic area seems to be important in this respect (Foidart, Silverin et al. 1998; Riters, Eens et al. 2000; Soma, Schlinger et al. 2003).

Thus, although it is accepted that testosterone plays a role in the organization and activation of song (Bolhuis and Gahr 2006) and territorial behaviour in general, it is still unclear to what extent testosterone facilitates these behaviours in species that sing and defend territories also outside the breeding season.

The first aim of this study was to describe the seasonal testosterone profile in a temperate zone songbird species, the black redstart *(Phoenicurus ochruros)*, and correlate it with territorial behaviour, song rate and structure, and changes in brain structure. We describe the expression pattern of androgen-, oestrogen receptor and aromatase mRNA in the forebrain song control nuclei and in diencephalic areas of male black redstarts, which are known only for a small number of songbird species so far (Metzdorf, Gahr et al. 1999). If there are seasonal differences in song output and structure, we expected males to have a larger HVC in spring than in autumn. As aromatase expression in the pre-optic area has been shown to play an important role in the expression of reproductive behaviours, we expected a higher expression of aromatase mRNA in that area during spring compared to the autumn territorial phase.

The second aim of this study was to test if seasonal changes in spontaneous song output and structure are facilitated by testosterone or its metabolites. To do so we implanted males with an androgen receptor blocker and an aromatase inhibitor in spring and in autumn and compared the song output and structure before and after implantation.

If testosterone activates song during the breeding season we expected that males produce more spontaneous song during spring than during non-breeding in autumn. Further, we then expected that blocking the actions of testosterone should reduce song output in spring, but not in autumn. If testosterone (of non-gonadal origin) activates song also during autumn we expect no seasonal difference in spontaneous song output and blocking the actions of testosterone should reduce the song output during both seasons. If testosterone does not activate song we expect no seasonal difference in the production of spontaneous song and no influence of blocking the actions of testosterone during spring and autumn.

Similar to other species, parts of the song of black redstarts contain repetitive elements and we have previously shown that males increase the number of these elements in an agonistic context (Apfelbeck et al., submitted). If testosterone changes the structure of song during the breeding season, we expected to find significant differences in song structure between spring and autumn. For example, males may sing a higher number of repetitive elements in spring compared to autumn. Blocking the actions of testosterone should then affect song structure during spring, but not in autumn. If testosterone changes the song structure only in spring applying these blockers should specifically reduce the number of repetitive elements in spring, but not in autumn. However, if testosterone (of non-gonadal origin) activates song structure also during autumn we expect no seasonal difference in song structure and blocking the actions of testosterone in both seasons should have an effect on song structure.

If testosterone does not regulate song structure we expect no seasonal difference in song structure and no influence of blocking the actions of testosterone during both seasons.

Methods

Male black redstarts defend a territory and sing during the breeding season. During late summer, when they moult, singing activity is very low but increases again in autumn just before migration (Weggler 2000; Nicolai 2005). Redstarts are socially monogamous and both males and females feed nestlings and fledged young (Landmann 1996; Draganoiu, Nagle et al. 2005).

Male black redstarts were caught and their territorial behaviour recorded between April 1 and June 12 and between September 19 and October 6, 2008, between July 3 and August 13, 2009 and between June 28 and July 31, 2010 in Upper Bavaria, Germany (N 47º, E 11º, 500-600 m above sea level). Some of the hormone samples and the behaviour in response to the simulated territorial intrusions were collected as part of a different study and are also published elsewhere (Apfelbeck and Goymann 2011, chapter 2). We have previously shown that male black redstarts have high testosterone levels at the beginning of the breeding season and very low levels outside the breeding season in autumn (Apfelbeck and Goymann 2011, chapter 2). In this study we complemented the profile by adding measurements from the whole breeding season. In this previous study we have also shown that although testosterone levels were low in

autumn, territorial behaviour was not significantly different from territorial behaviour in spring (Apfelbeck and Goymann 2011, chapter 2).

Simulated territorial intrusion

To elicit a territorial response we placed a stuffed decoy into the centre of a territory and played back black redstart song using five different playbacks in random order (see Apfelbeck and Goymann 2011, chapter 2). We recorded the following behaviours of the territory owner for 10 min: (1) latency to respond to the STI either by singing or approaching the decoy, (2) the first time the male was within 5 m of the decoy, (3) the time the male spent within 5 m of the decoy, and (4) the time the territory owner had its feathers fluffed, which is a typical threat posture of male black redstarts (Landmann and Kollinsky 1995). Furthermore, we noted whether the male attacked the decoy or sang at any time during the STI.

Capture and blood sampling

Males were caught either after the simulated territorial intrusion experiments or passively while searching for food with mealworm-baited ground traps. We have shown previously that simulated territorial intrusions do not increase plasma testosterone levels in male black redstarts (Apfelbeck and Goymann 2011, chapter 2). Immediately upon capture, a blood sample (~120 µl) was taken after venipuncture from the wing vein (178 ± 112 s), and collected into heparinised capillaries. In addition, each male was banded with a numbered aluminium ring (Vogelwarte Radolfzell) and a combination of two colour rings. All experimental procedures were approved by the governmental authorities of Upper Bavaria.

Recording and analysis of spontaneous song

Adult (≥ 2 years) territorial male black redstarts were recorded with a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660 (sampling frequency: 44.1 kHz; resolution: 16 bit) in spring 2009 (April 9 - 27) and a different set of individuals in autumn 2009 (September 22 – October 7).

After obtaining samples of spontaneous song, the individuals (spring n = 20, autumn: n = 12) were caught and implanted either with a placebo pellet or two time-release pellets (Innovative Research of America, Sarasota, FL) containing flutamide (an androgen receptor blocker) and letrozole (an aromatase inhibitor) blocking thus both direct and indirect effects of testosterone on behaviour (for details see Apfelbeck et al., in prep., chapter 5). Three days after implantation spontaneous song was recorded again.

The song was analysed using Avisoft-SASLab Pro software, version 4.51. Recordings were visualized in spectrograms (settings: sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50 %). We determined the number of songs by visual inspection and selected songs of sufficient quality (low background noise) for further sound analysis. Each song of black redstarts can be divided into three distinct parts (part A, B and C, e.g. Cucco and Malacarne 1999, chapter 4 and 5) with a pause of varying length between part A and B. We measured the duration of part A, B, C, the total song and the duration of pauses between A and B (see Apfelbeck et al., submitted, chapter 4). We counted the number of elements of part A and C (mean of max. 20 songs). We also

determined the frequency bandwidth and the maximum frequency of part A, B and C using the automatic parameter measurement function (threshold -20 dB) in Avisoft (mean of max. 10 renditions of high-quality songs).

Plasma separation and hormone analysis

Plasma was immediately separated by centrifugation with a Compur Minicentrifuge (Bayer Diagnostics). The amount of plasma was measured with a Hamilton syringe and stored in 500 µl ethanol (Goymann, Schwabl et al. 2007). After returning from the field samples were stored at -80 °C. Testosterone concentration was determined by direct radioimmunoassay (RIA, following Goymann, Geue et al. 2006; Apfelbeck and Goymann 2011). Samples were measured in duplicates in four assays. Mean ± SD efficiency of the extraction with dichloromethane was 99 ± 0.01 %, 92 % ± 0.1 %, 91 % ± 0.1 %, 88 % ± 0.1 % respectively. The lower limit of detection of the assay was determined as the first value outside the 95 % confidence intervals for the zero standard (B_{max}) and was 6.7, 6.6, 6.9 and 8.0 pg/tube respectively. The intra-assay coefficients of variation were 4.7 %, 4.2 %, 2.9 % and 7.9 % respectively. The inter-assay variation was 3.9 %. As the testosterone antibody shows significant cross-reactions with 5a-dihydrotestosterone (44 %) our measurements may include a fraction of this additional androgen.

Tissue collection

Brains were collected between April 14 and 28 in 2008 (n = 8) and between September 21 and 24 in 2009 (n = 8). Upon capture, birds were immediately killed by decapitation and brains dissected out of the skull, frozen on dry ice and

stored at -80 °C until further processed. We also recorded the length and width of the left and right testis and calculated testis volume as the volume of an oval body ($4/3*\pi*(width/2)^2*(length/2)$). Frozen brains were cut into 20 µm sagittal sections on a cryostat microtome (Leica Microsystems GmbH, Wetzlar, Germany) and collected on Superfrost object slides (Menzel GmbH, Braunschweig, Germany) in five parallel series. One series of brain sections was selected for Nissl staining and used to provide anatomical landmarks for later interpretation of in-situ hybridization results. The remaining series were used for in situ hybridization of adjacent sections for androgen receptor (AR), oestrogen receptor α (ERα) and aromatase.

In-situ hybridization

Riboprobes were synthesized from cDNA previously cloned from zebra finch AR and ERα mRNA (Gahr and Metzdorf 1997) and canary aromatase mRNA (Metzdorf, Gahr et al. 1999). Antisense and sense ^{35}S-CTP-labelled probes were transcribed from the T7 and SP6 promoter region of a pGEM7Zf+ vector using the Riboprobe System (Promega, Madison, WI).

Brain sections were fixed in a 4 % formaldehyde solution in phosphate-buffered saline (PBS; 0.01M; pH 7.4) for 5 min, washed in DEPC-treated PBS , and incubated in 0.25% acetic anhydride in ethanolamine (TEA; 0.1M; pH 8.0) for 10 min to reduce non-specific binding. After a washing step in 2x standard saline citrate (SSC), sections were dehydrated in serially increasing percentages of ethanol, and left to dry at room temperature. Sections were hybridized under a cover slide with ^{35}S-CTP-labeled *sense or* antisense riboprobes (0.4×10^6 cpm /

slide) in hybridization buffer with 50 % formamide and 10 % dextran sulfate overnight at 55 °C. After hybridization, slides were immersed in 2X SSC at room temperature to remove the cover slides and incubated in RNase A (20 ug/ml) for 30 min at room temperature. Sections were then consecutively washed for 30 min in 2x SSC at 50 °C, 0.2x SSC at 55 °C, and 0.2x SSC at 60 °C, dehydrated in ethanol containing 0.3 M ammonium acetate, and dried for 1 hour at room temperature. Finally, slides were exposed to Kodak BioMax MR film (Sigma-Aldrich Co., St. Louis, MO) in lightproof boxes for 3 weeks at room temperature, developed in Kodak D-19 developer, washed in tap water, and fixed with Kodak fixer.

Brain data analysis

Data analysis was carried out similarly as described in (Voigt, Ball et al. 2009). Autoradiograms were scanned with an Epson scanner using SilverFast Ai software as 16 bit grey values and with a resolution of 2400 dpi for later analysis in ImageJ. The system was calibrated by scanning a calibrated optical density step tablet (part #T2115, Stouffer Graphic Arts Equipment Co., Mishawaka, USA) and a calibration curve was calculated based on the Rodbard function in ImageJ. All autoradiogram images were saved in ImageJ with this calibration. HVC volumes were estimated based on AR expression (see also Gahr and Metzdorf 1997; Fusani, Van't Hof et al. 2000). For each AR-labelled brain section we delineated HVC, summed the area measurements and multiplied them with 100 µm (interval between sections). To quantify AR, ERα and aromatase mRNA expression levels in the pre-optic area we measured the optical density. The

location of the pre-optic area was determined manually by overlapping adjacent sections labelled for AR, ERα, aromatase and Nissl. The pre-optic area can be found in medial sections of the brain and lies ventral from the anterior commissure. Optical density was measured in two different ways, using an ellipsoid with fixed dimensions for all sections and individuals, and again using an ellipsoid covering most of the stained area (therefore with changing size between individuals). To control for background staining the optical density in a control area just adjacent to the pre-optic area was subtracted from the value for receptor expression. Optical density measures were averaged across all sections that were labelled for the respective mRNA. All measurements were carried out blind to the seasonal group of the bird.

Statistical analysis

Data were analysed in R (Development Core Team 2009) using linear models and were transformed, if necessary, to meet assumptions of equality and normality of variances. Brain data did not meet these assumptions and were therefore tested for seasonal differences using Mann-Whitney-U-tests for non-parametric independent samples.

Results

Seasonal testosterone profile

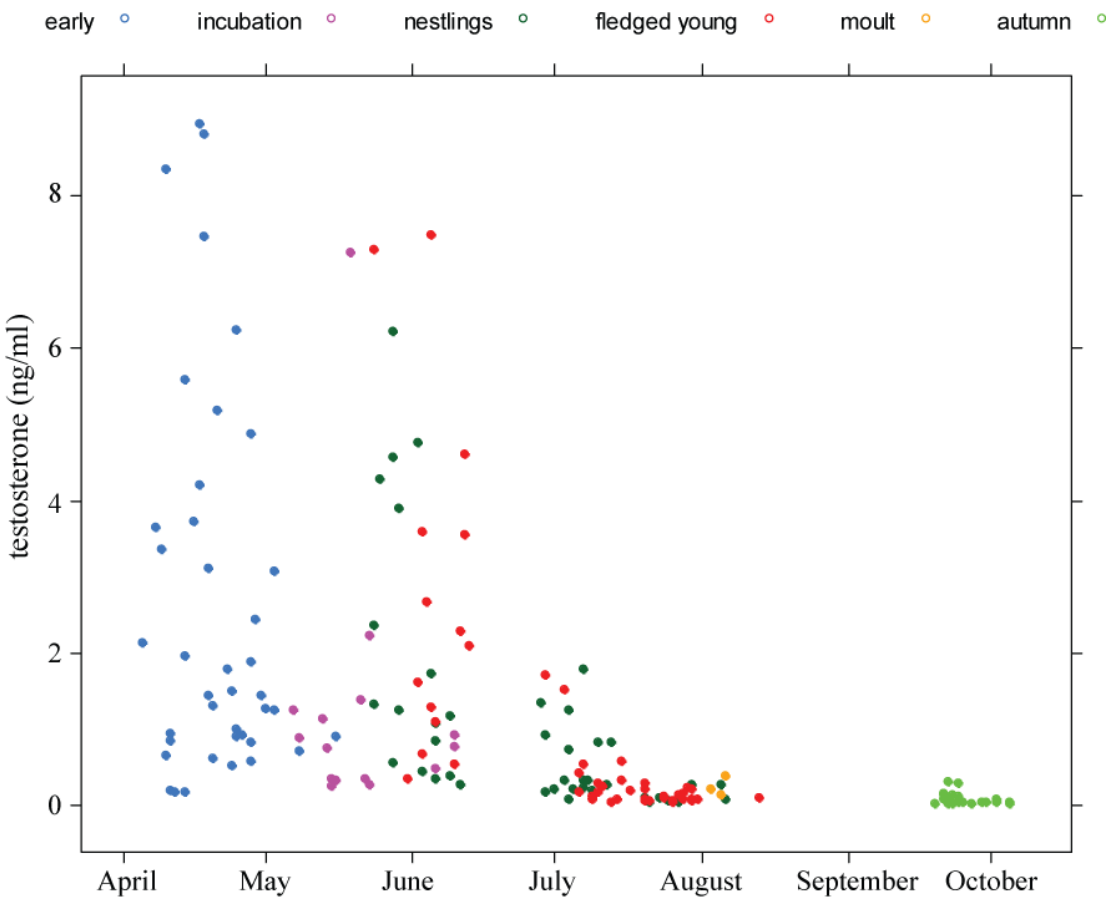

Figure 1: Post-capture testosterone levels (ng/ml) of male black redstarts caught during different times of the year during breeding season and in autumn (autumn territorial phase). Testosterone levels in autumn were below 80 pg/ml. Each individual is represented only once.

Testosterone levels of males caught during various times of the year differed significantly ($F_{5,133} = 21$, $p < 0.0001$, Fig. 1). A priori set contrasts revealed that levels during the incubation phase of the first brood were not significantly

different from testosterone levels during territory establishment ($t = 0.4$, $p = 0.7$). During all other phases of the life-cycle testosterone levels were significantly lower than during territory establishment (nestlings (first and second brood combined): $t = -3.4$, $p = 0.001$; fledglings (first and second brood combined): $t = -6.0$, $p < 0.0001$; moult: $t = -2.5$, $p = 0.01$; autumn: $t = -9.4$, $p < 0.0001$). Comparing only breeding stages, testosterone levels also significantly differed ($F_{2,118} = 69$, $p < 0.0001$). Males caught during their second brood had significantly lower testosterone levels than males caught during territory establishment ($t = -9.1$, $p < 0.0001$), but not males caught during their first brood ($t = 0.8$, $p = 0.4$). Testes in autumn were completely regressed (e.g. volume left testis (mean +/- 95 % CI): spring: 76.1 +/- 14.0 mm^3, n = 8, autumn: 0.5 +/- 0.5 mm^3, n = 8, $U = 0$, $p = 0.0002$).

Behavioural response to STI

The response of territorial males to simulated territorial intrusions differed only slightly between breeding stages and seasons. Time spent within 5 m of the decoy and time spent with their feathers fluffed neither differed between territory establishment and the parental phase nor between territory establishment and autumn (time in 5 m: $F_{2, 56} = 0.4$, $p = 0.6$; fluffing (%): $F_{2, 56} = 0.7$, $p = 0.5$; Fig. 2). However, males approached the decoy faster during territory establishment than when they were feeding young of their first brood ($t = 3.1$, $p = 0.003$, Fig. 2). In contrast, they approached the dummy as fast in autumn as during territory establishment ($t = 1.4$, $p = 0.2$; overall model: $F_{2, 56} = 5.0$, $p = 0.01$, Fig. 2). Also, the number of individuals that sat on the cage of the decoy and tried to attack the

decoy did not differ between breeding stages and seasons (Table 1). However, fewer individuals sang during the simulated territorial intrusion in autumn than during the breeding stages (Table 1).

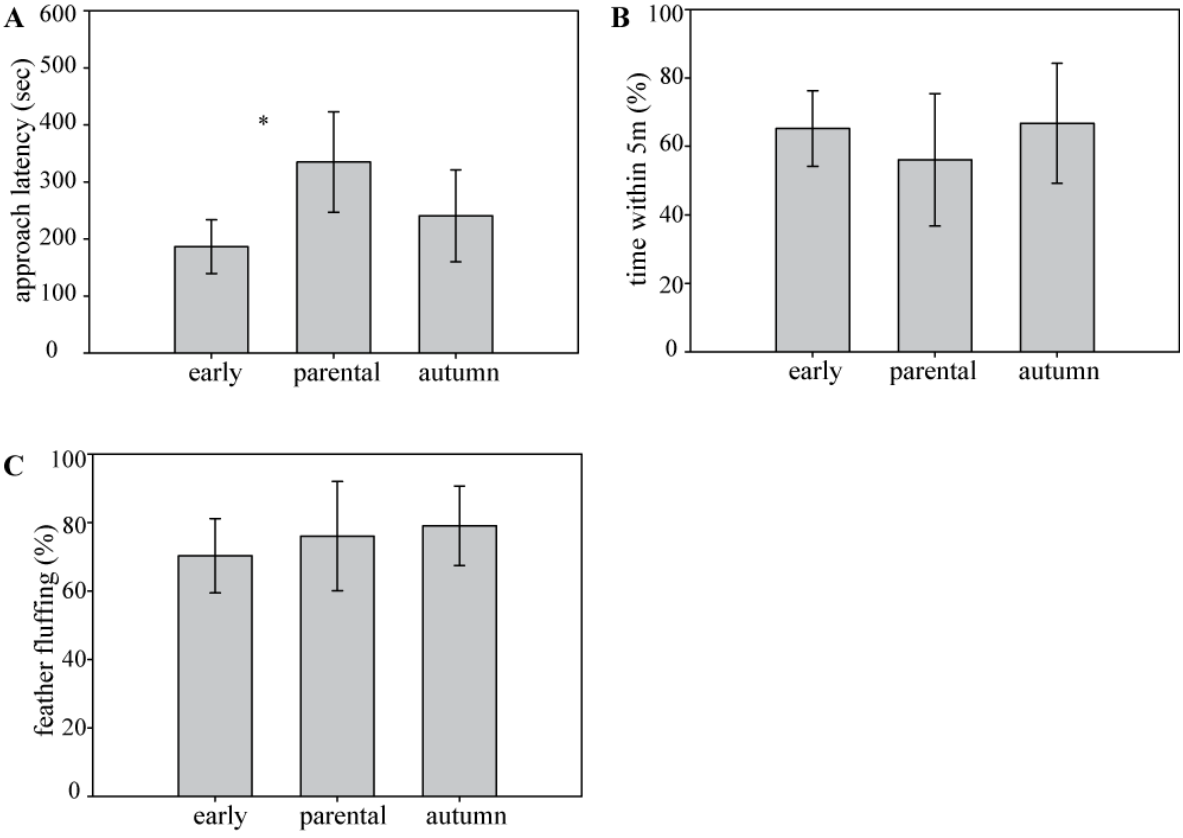

Figure 2: Latency to approach (within 5 m, A), time spent within 5 m (as % of time seen, B) and time spent feather fluffing (as % of time seen, C) in response to a simulated territorial intruder. Early breeding season: n = 31; feeding of nestlings or fledglings of the first brood (parental): n = 11; autumn territorial phase: n = 17. Bars represent means +/- 95 % CI. Asterisks indicate significant differences between breeding stages (*: $p < 0.05$).

Table 1. Number of individuals that sang and sat on the cage around the decoy at any time during the STI experiment until capture. P-values in brackets refer to a priori set contrasts: territory establishment versus parental phase / territory establishment versus autumn.

	early	**parental phase**	**autumn**	**Fisher test**
song yes	26	9	7	p = 0.007; (p = 1 / p = 0.004)
song no	5	2	10	
song yes *	19	4	1	p < 0.003; (p = 0.18 / p < 0.001)
song no *	12	7	16	
cage yes	10	3	7	p = 0.8
cage no	21	8	10	
cage yes *	6	2	5	p = 0.7
cage no *	25	9	12	

*Number of individuals during the first 10 min of the STI before traps were opened.

Expression patterns of androgen receptor (AR), oestrogen receptor (ERα) and aromatase

AR-, ERα- and aromatase mRNA expression patterns were very similar to expression patterns found in other songbird species and were similar in spring and in autumn.

Telencephalon: Song control system

The expression pattern of AR-, ERα- and aromatase mRNA in the song control nuclei was similar to that found in other songbirds (Gahr and Metzdorf 1997; Metzdorf, Gahr et al. 1999) and did not differ between seasons (Table 2). Dense

androgen receptor mRNA staining was found in the song control nuclei HVC, lMAN, mMAN and in the arcopallium (for an example see Fig. 3). ERα mRNA expression was only found in HVC and was much weaker than AR expression in HVC. Furthermore, expression of ERα mRNA was restricted to the so called paraHVC (Gahr and Metzdorf 1997). Thus, ERα mRNA expression was only found in medial sections when HVC was blending into the caudomedial neostriatum (NCM) and in most individuals overlapped with AR expression in a few sections. Aromatase expression was not found in any of the song control nuclei. However, dense staining for aromatase mRNA occurred in the NCM (Shen, Schlinger et al. 1995). Similar to canaries, aromatase and ERα expression in the NCM did not overlap (Metzdorf, Gahr et al. 1999), but aromatase and AR expression did. ERα in the NCM was especially expressed around the lateral ventricle.

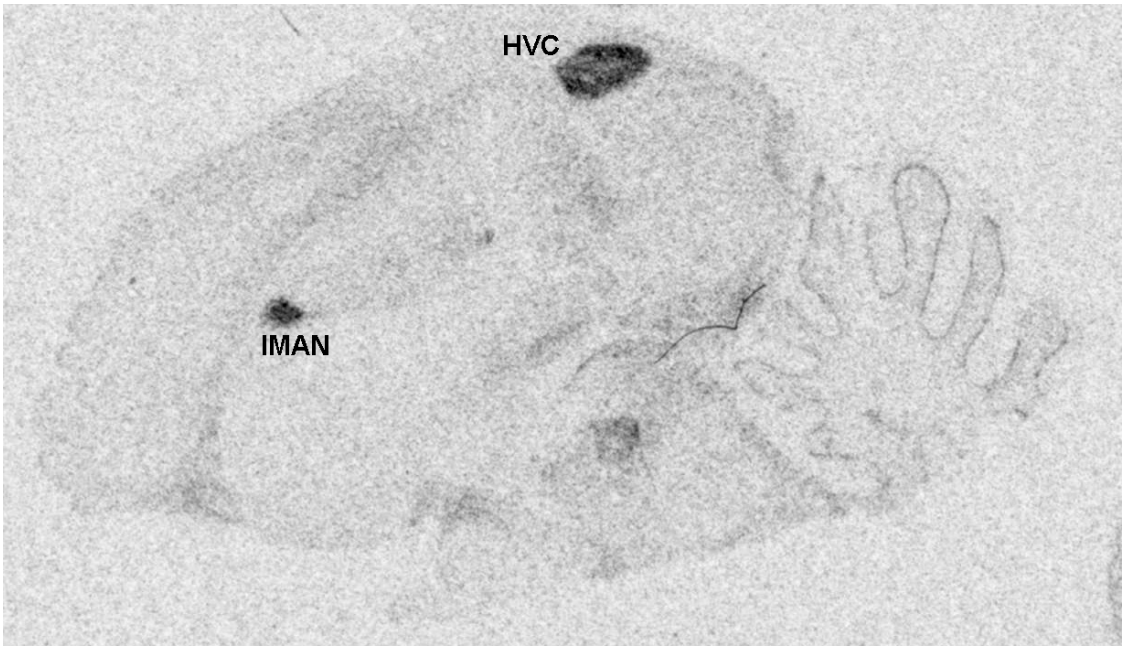

Figure 3. Example for androgen receptor mRNA expression in two song control nuclei in spring. HVC…proper name, IMAN…lateral nucleus magnocellularis.

Diencephalon: pre-optic and hypothalamic areas

During both seasons co-expression of AR, ERα and aromatase was found in the pre-optic area. AR, ERα and aromatase were also co-expressed in the nucleus lateralis hypothalami posterioris (PLH, for an example see Fig. 4), but similar to other species no ERα expression was found in the nucleus medialis hypothalami posterioris (PMH, e.g. Fig. 4B, Metzdorf, Gahr et al. 1999; Fusani, Van't Hof et al. 2000). The tuberal region contained especially dense staining for ERα mRNA (e.g. Fig. 4B), to a lesser degree for AR mRNA (e.g. Fig. 4A) and weak staining for aromatase mRNA (e.g. Fig. 4C). Further staining for AR-, ERα and aromatase mRNA was found in the bed nucleus of the stria terminalis (BNST, Table 2).

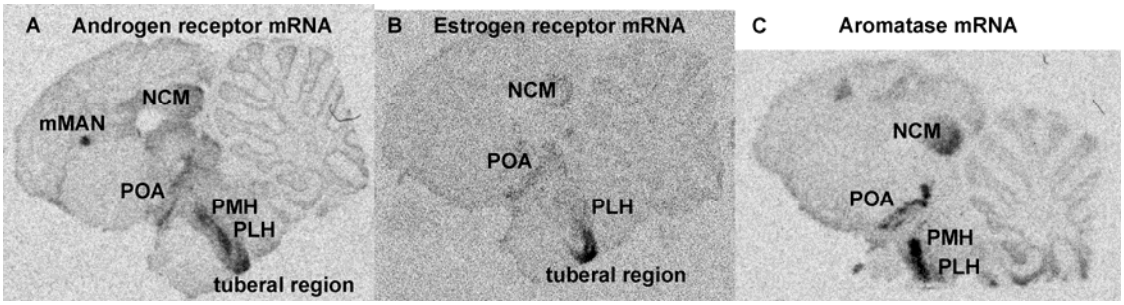

Figure 4. Example for androgen receptor (A), estrogen receptor (B) and aromatase (C) mRNA expression in spring. mMAN… medial nucleus magnocellularis, NCM…caudo-medial neostriatum, POA… pre-optic area, PMH…nucleus posterioris hypothalami medialis, PLH… nucleus posterioris hypothalami lateralis.

Table 2. Brain areas in the telencephalon and diencephalon expressing androgen receptor (AR), estrogen receptor (ERα) and or aromatase (ARO) depending on season. lMAN… lateral nucleus magnocellularis, mMAN… medial nucleus magnocellularis, NCM…caudo-medial neostriatum, POA… pre-optic area, PMH…nucleus posterioris hypothalami medialis, PLH… nucleus posterioris hypothalami lateralis, BNST…bed nucleus of the stria terminalis.

Brain area	**AR spring**	**AR autumn**	**ARO spring**	**ARO autumn**	**ERα spring**	**ERα autumn**
telencephalon						
HVC	yes	yes	no	no	yes	yes
lMAN	yes	yes	no	no	no	no
mMAN	yes	yes	no	no	no	no
arcopallium	yes	yes	no	no	no	no
NCM	yes	yes	yes	yes	yes	yes
diencephalon						
POA	yes	yes	yes	yes	yes	yes
PMH	yes	yes	yes	yes	no	no
PLH	yes	yes	yes	yes	yes	yes
Tuberal region	yes	yes	yes, but weak	yes, but weak	yes	yes
BNST	yes	yes	yes	yes	yes	yes

HVC volume

HVC volume determined by androgen receptor expression tended to be higher in autumn than in spring (mean +/- 95 % CI: spring: 0.6 +/- 0.1 mm^3, n = 8, autumn: 0.8 +/- 0.08 mm^3, n = 8; t = 2.0, df = 12, p = 0.07).

Optical density in pre-optic area

As both methods used to measure optical density produced similar results, we only present results for the second one of the methods (see methods; ellipsoid fitted to area with staining). Aromatase mRNA expression in the pre-optic area was significantly higher spring than in autumn (U = 45; p = 0.007, n = 8 per season, Fig. 3). Expression of estrogen receptor mRNA (U = 12, p = 0.1) and androgen receptor mRNA (U = 26.5, p = 0.9) did not differ significantly between seasons.

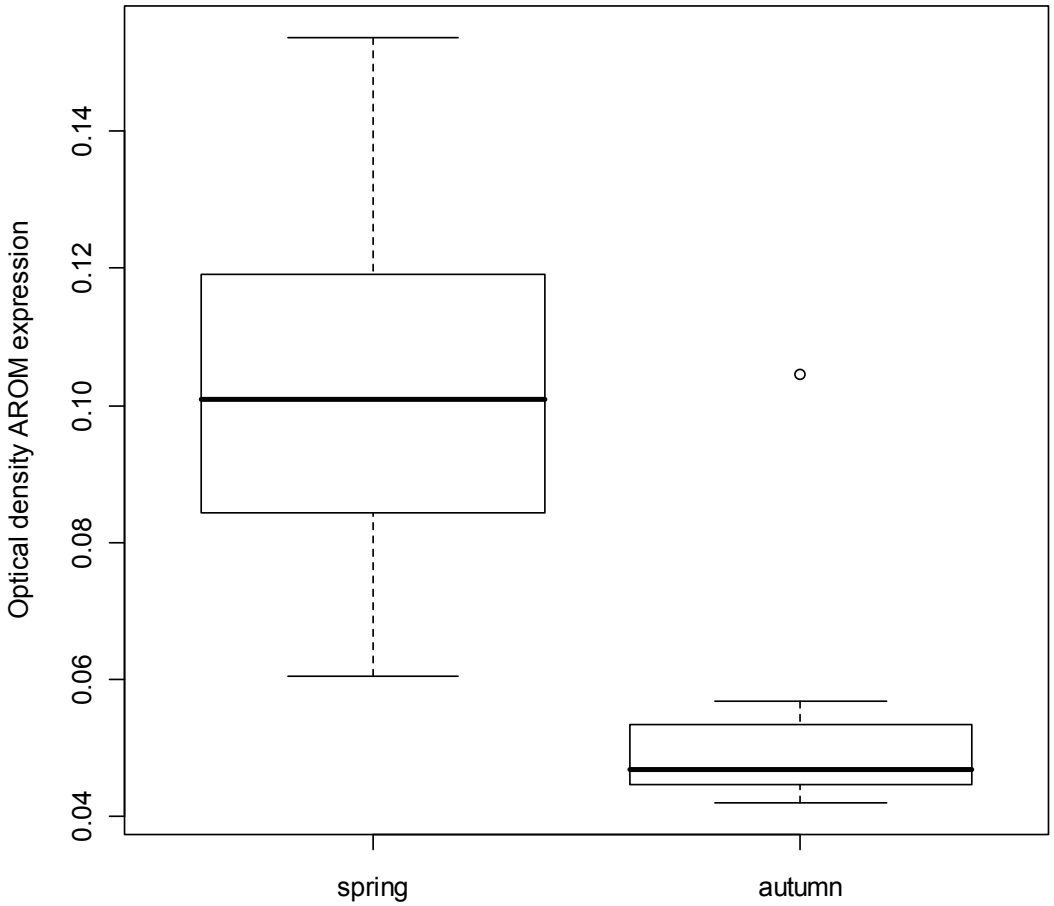

Figure 3. Box plots comparing the optical density of aromatase expression in the pre-optic area between spring and autumn. Data are presented as medians and 95 % CIs.

Spontaneous song: seasonal differences

Territorial male black redstarts were singing significantly more songs with shorter pauses between songs during bouts of spontaneous song in autumn than in

spring (Table 3). However, songs sang spontaneously during spring had more elements in part A and C than those sang in autumn (Table 3, Fig. 4). All other structural song parameters did not differ significantly between seasons (Table 3).

Spontaneous song: treatment effects

Implantation of blockers did not significantly change any of the song parameters in spring or in autumn (Table 4 and 5). However in spring, blocker-implanted males tended to sing fewer songs and longer pauses between songs during bouts of spontaneous song three days after than before implantation (Table 4). There was no significant change in control males after implantation of a placebo (Table 4). In autumn, implantation of blockers or a placebo had no effect on song rate or the duration of pauses between songs (Table 5).

Table 3. Comparison of song parameters during bouts of spontaneous song between spring and autumn.

song parameter	t-statistic (df=30)	p-value
song rate	**-3.9**	**0.0005**
pauses in bouts	**3.2**	**0.004**
duration A	-0.7	0.5
duration B	-0.6	0.6
duration C	1.8	0.09
pause A - B	0.4	0.7
nr of elements in A	**-2.0**	**0.05**
nr of elements in C	**-2.8**	**0.009**
max frequency A	0.6	0.6
freq bandwidth A	-0.5	0.6
max frequency B	-0.1	0.9
freq bandwidth B	-0.4	0.7
max frequency C	-1.7	0.09
bandwidth C	-1.9	0.07

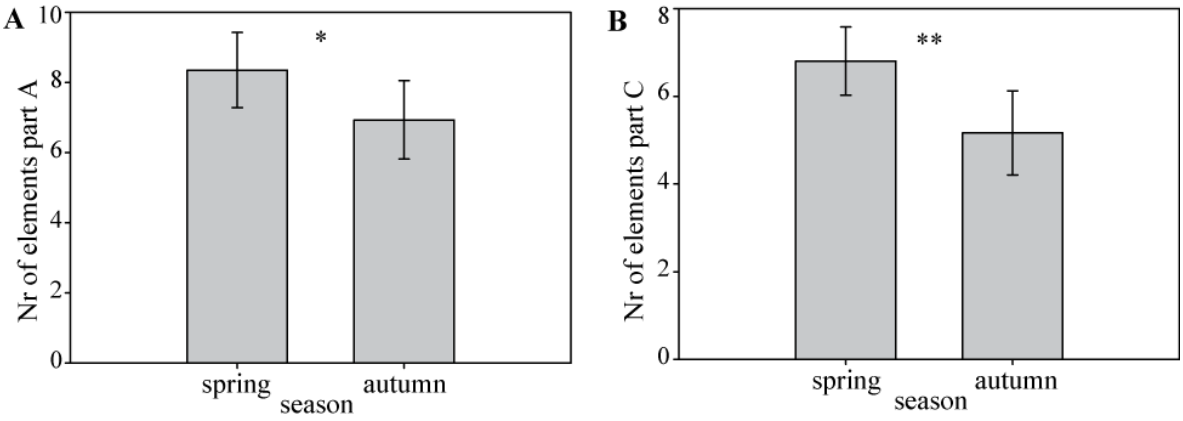

Figure 4: Number of elements sang in part A (A) and C (B) spontaneously depending on season. Spring: n = 20; autumn territorial phase: n = 12. Bars represent means +/- 95 % CI. Asterisks indicate significant differences between seasons (*: p=0.05; **: p<0.01).

Table 4. Comparison of song parameters during bouts of spontaneous song before and after implantation (implant) of a placebo or blockers (treatment) in spring.

song parameter	treatment	implant	interaction
song rate	F1,18=2.8, p=0.1	**F1,18=7.8, p=0.01**	F1,18=1.9, p=0.2
pauses in bouts	F1,18=0.2, p=0.7	**F1,18=10.0, p=0.005**	**F1,18=15.6, p=0.0009**
song duration	F1,18=0.06, p=0.8	F1,18=2.1, p=0.2	F1,18=0.9, p=0.3
duration pause A-B	F1,18=0.009, p=0.9	F1,18=0.008, p=0.9	F1,18=1.0, p=0.3
duration A	F1,18=2.9, p=0.1	F1,18=1.5, p=0.2	F1,18=0.09, p=0.8
duration B	F1,18=0.004, p=1.0	F1,18=1.0, p=0.3	F1,18=0.09, p=0.8
duration C	F1,18=0.2, p=0.7	F1,18=1.4, p=0.2	F1,18=2.1, p=0.2
no of elements in A	F1,18=1.5, p=0.2	F1,18=3.1, p=0.09	F1,18=1.9, p=0.2
no of elements in C	F1,18=0.5, p=0.5	F1,18=0.3, p=0.6	F1,18=2.2, p=0.2
freq bandwidth A	F1,18=0.8, p=0.4	F1,18=0.01, p=0.9	F1,18=2.5, p=0.1
max frequency A	F1,18=0.09, p=0.8	F1,18=0.7, p=0.4	F1,18=3.4, p=0.08
freq bandwidth B	F1,18=0.005, p=0.9	F1,18=1.1, p=0.3	F1,18=3.3, p=0.09
max frequency B	F1,18=1.1, p=0.3	F1,18=0.0, p=0.9	F1,18=0.1, p=0.8
bandwidth C	F1,18=0.1, p=0.7	F1,18=1.9, p=0.2	F1,18=0.06, p=0.8
max frequency C	F1,18=0.2, p=0.6	F1,18=0.0, p=1.0	F1,18=0.2, p=0.7

Table 5. Comparison of song parameters during bouts of spontaneous song before and after implantation (implant) of a placebo or blockers (treatment) in autumn.

	treatment	**implant**	**interaction**
song rate	F1,10=4.9, p=0.05	F1,10=0.8, p=0.4	F1,10=0.3, p=0.6
pauses in bouts	F1,10=0.2, p=0.6	F1,10=1.0, p=0.3	F1,10=0.06, p=0.8
song duration	F1,10=0.2, p=0.7	F1,10=0.2, p=0.7	F1,10=0.7, p=0.4
pause A - B	F1,10=0.8, p=0.4	F1,10=0.002, p=1.0	F1,10=0.3, p=0.6
duration A	F1,10=0.3, p=0.6	F1,10=0.1, p=0.7	F1,10=0.02, p=0.9
duration B	F1,10=0.5, p=0.5	F1,10=0.7, p=0.4	F1,10=0.4, p=0.5
duration C	F1,10=0.02, p=0.9	F1,10=0.4, p=0.6	F1,10=0.1, p=0.7
no of elements in A	F1,10=0.5, p=0.5	F1,10=0.4, p=0.6	F1,10=0.1, p=0.8
no of elements in C	F1,10=0.1, p=0.7	F1,10=2.3, p=0.2	F1,10=0.4, p=0.5
bandwidth A	F1,10=0.2, p=0.6	F1,10=0.05, p=0.8	F1,10=0.2, p=0.7
max frequency A	F1,10=0.0, p=1.0	F1,10=1.0, p=0.4	F1,10=0.2, p=0.6
freq bandwidth B	F1,10=2.2, p=0.2	F1,9=2.5, p=0.1	F1,9=2.1, p=0.2
max frequency B	F1,10=3.5, p=0.09	F1,9=4.1, p=0.07	F1,9=3.1, p=0.1
freq bandwidth C	F1,10=0.1, p=0.8	F1,10=0.0, p=1.0	F1,10=0.01, p=0.9
max frequency C	F1,10=0.0, p=0.9	F1,10=0.1, p=0.7	F1,10=0.1, p=0.7

Table 6. Summary of physiological, morphological and behavioral results comparing breeding and non-breeding territoriality of male black redstarts.

	breeding	**non-breeding**
testosterone	high	low
testes	large	regressed
response to STI		
non-vocal	strong	strong
song rate	high	low
spontaneous song		
song rate	high	high
song structure	more elements in A and C	fewer elements in A and C
brain anatomy		
HVC	large	large
aromatase in pre-optic area	strong	weak

Discussion

Male black redstarts defend territories during breeding and in autumn, i.e. after having finished moult and before migrating south and in general breed on these territories in the following spring (Weggler 2000; Apfelbeck and Goymann 2011). During the autumn territorial phase, testes were regressed and circulating testosterone levels were very low. Further, during autumn testosterone did not increase in response to territorial or physiological (GnRH) challenges (Apfelbeck

and Goymann 2011). Despite these low levels of testosterone, males aggressively defended their territories just as vigorously as they did during territory establishment in spring. Furthermore, although the likelihood of song in response to a simulated territorial intrusion was lower in autumn than in spring, the spontaneous song rate was even higher in autumn. However, males sang more elements in the trill-like parts of their song during breeding. HVC size as delimited by androgen receptor mRNA expression did not differ between seasons, but aromatase expression in the pre-optic area was higher during spring than in autumn. Thus, the relationship between testosterone and territorial behaviour in male black redstarts is complex: testosterone does not seem to regulate non-vocal territorial behaviours, but the hormone may be involved in the regulation of song (Table 6). Testosterone may influence song output and it may stimulate males to produce more repetitive elements in their song during breeding compared to non-breeding.

In previous studies we have shown that it is very unlikely that testosterone facilitates the overall territorial response of male black redstarts. Instead, it seems to emphasize vocal- over non-vocal territorial behaviours during the breeding season in this species (Apfelbeck et al., in prep., chapter 5). The correlational evidence from this study confirms this view: males responded to a simulated territorial intruder with an equally high intensity and were as likely to attack the intruder during non-breeding as during breeding. However, the likelihood to respond with song to the experimental intrusion was significantly reduced during non-breeding compared to breeding. Our data corroborate similar

findings in other bird species that defend territories outside the breeding season: males can respond to intruders and defend a territory even with low circulating testosterone levels (Burger and Millar 1980; Logan and Wingfield 1990; Schwabl and Kriner 1991; Wingfield 1994; Soma, Sullivan et al. 1999; Canoine and Gwinner 2002; Landys, Goymann et al. 2010; Hau and Beebe 2011) and sing at a high rate also in autumn (Riters, Eens et al. 2000).

Further evidence that testosterone may shift the focus of the territorial response to song related parameters during breeding (Apfelbeck et al., in prep., chapter 5) comes from our experimental study on spontaneous song behaviour: treatment with an anti-androgen and an aromatase inhibitor increased the duration of pauses between songs during bouts of spontaneous song during breeding but not during non-breeding. Similarly, studies that experimentally increased testosterone during the breeding season could induce males to sing more (Ketterson, Nolan et al. 1992; Van Duyse, Pinxten et al. 2002; Ritschard, Laucht et al. 2011, but see Kunc, Foerster et al. 2006). In most songbirds, song is not only important for territory defence, but also for the attraction and stimulation of mates. For example, in European starlings *(Sturnus vulgaris)*, the presence of a female induces an increase in song rate in males during breeding but not outside the breeding season (Riters, Eens et al. 2000). Furthermore, in many species song rate is highest when females are fertile (Mace 1987; Gil and Gahr 2002). Experimentally administered testosterone stimulates males to maintain a high song rate also later during the breeding season (Silverin 1980; Ketterson, Nolan et al. 1992; Van Duyse, Pinxten et al. 2002). Furthermore,

testosterone is not only thought to activate territorial behaviour and song, but has also been suggested to balance the trade-off between mating effort and parental care. Accordingly, experimentally elevated testosterone decreases feeding effort in some species (Hegner and Wingfield 1987; Ketterson, Nolan et al. 1992). Male black redstarts that were implanted with blockers were caught during the first four weeks of the breeding season. Unfortunately, we were not able to assess the exact breeding stage of these males, however, their females were most likely starting to build nests and also lay eggs. Similar to others species, song output in black redstarts is highest around the time of mating (Landmann 1996). Thus, the increase in pause duration between songs after blocker implantation may indicate that males invested less into behaviours associated with mating. Also the seasonal testosterone data indicate that testosterone may facilitate mating behaviours in male black redstarts. Testosterone levels during territory establishment at the beginning of the breeding season and during the parental stages of the first brood were not significantly different. In redstarts broods overlap to a large degree and couples start a second clutch already while still feeding their first brood. However, during the nestling and fledgling stages of the last brood, when no more matings occurred, testosterone levels were significantly lower than during the first brood.

These changes in the relative importance of mating behaviours, such as song output, may be facilitated by the conversion of testosterone to oestradiol in the pre-optic area: aromatase mRNA in that area was expressed more at the beginning of the breeding season than in autumn. For example, in European

starlings the increase in song rate in response to females and other courtship behaviours correlate with a high activity of aromatase in that region (Riters, Eens et al. 2000; Pintér, Péczely et al. 2011).

The correlational data suggest that the number of elements in parts of the song of male black redstarts may be regulated by testosterone, because the number of repetitive elements in their song was higher during breeding compared to non-breeding. The number of repetitive elements in parts of the song or the length of trills has been shown to change seasonally in other species as well (Smith, Brenowitz et al. 1997; Leitner, Voigt et al. 2001; Voigt and Leitner 2008). Furthermore, these repetitive elements seem to be a relevant cue for females to assess male quality (Vallet, Kreutzer et al. 1997; Draganoiu, Nagle et al. 2002; Ballentine, Hyman et al. 2004; Ballentine 2009). Seasonal changes in syllable repetition rates may be facilitated by changes in the sensitivity for androgens and oestrogens in the song control nucleus HVC (Gahr and Metzdorf 1997). However, in male black redstarts the seasonal decrease in repetitive elements in some parts of the song does not seem to depend on testosterone or its conversion to oestrogens: the treatment with blockers had no significant effect on the number of elements produced. In addition, previous studies in male black redstarts have shown that males increased the number of elements in the respective parts of their song in an agonistic context both in the breeding and in the non-breeding season and independent of treatment with blockers (Apfelbeck et al., submitted, chapters 4 and 6). Thus, seasonal changes in element numbers during bouts of spontaneous song are probably independent of seasonal

changes in circulating testosterone levels or seasonal changes in brain sensitivity for sex steroids in male black redstarts. In other species seasonal changes in trill rate were correlated with changes in testosterone levels and HVC size (Smith, Brenowitz et al. 1997). Furthermore, HVC size is thought to depend at least partly on circulating testosterone levels (Sartor, Balthazart et al. 2005). Thus, it has been suggested that the production of fast trills may depend on testosterone-dependent seasonal changes in HVC size (Smith, Brenowitz et al. 1997). However, the correlational and experimental evidence from male black redstarts does not support this view as HVC size did not vary with season in this species.

Conclusions

In male black redstarts correlational evidence for a relationship between testosterone and territorial behaviour is rather weak, as males responded to a simulated territorial intrusion with a high intensity and sang at a high song rate also in autumn when testosterone levels were low. However, this and our previous studies suggest that the function of testosterone in male black redstarts may be two-fold. First, testosterone may shift the emphasis of the territorial response towards behaviours potentially associated with mating. In black redstarts song seems to be important in that respect. Second, some components of the vocal territorial response may be directly activated by testosterone or oestrogens during the breeding season, because frequency-related changes in song structure in response to a simulated territorial intruder were inhibited in males implanted with an anti-androgen and an aromatase inhibitor (Apfelbeck et

al., submitted). These seasonal changes in the role of song in the territorial response of black redstarts may be regulated by conversion of testosterone to oestradiol in the pre-optic area.

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PROCEEDINGS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Ignoring the challenge? Male black redstarts (*Phoenicurus ochruros*) do not increase testosterone levels during territorial conflicts but they do so in response to gonadotropin-releasing hormone

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Ignoring the challenge? Male black redstarts (*Phoenicurus ochruros*) do not increase testosterone levels during territorial conflicts but they do so in response to gonadotropin-releasing hormone

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Competition elevates plasma testosterone in a wide variety of vertebrates, including humans. The 'challenge hypothesis' proposes that seasonal peaks in testosterone during breeding are caused by social challenges from other males. However, during experimentally induced male–male conflicts, testosterone increases only in a minority of songbird species tested so far. Why is this so? Comparative evidence suggests that species with a short breeding season may not elevate testosterone levels during territory defence. These species may even be limited in their physiological capability to increase testosterone levels, which can be tested by injecting birds with gonadotropin-releasing hormone (GnRH). We studied two populations of black redstarts that differ in breeding altitude, morphology and the length of their breeding season. Unexpectedly, males of neither population increased testosterone in response to a simulated territorial intrusion, but injections with GnRH resulted in a major elevation of testosterone. Thus, black redstarts would have been capable of mounting a testosterone response during the male–male challenge. Our data show, for the first time, that the absence of an androgen response to male–male challenges is not owing to physiological limitations to increase testosterone. Furthermore, in contrast to comparative evidence between species, populations of black redstarts with a long breeding season do not show the expected elevation in testosterone during male–male challenges.

Keywords: GnRH; challenge hypothesis; number of broods hypothesis; breeding season length; territorial aggression; androgen responsiveness

1. INTRODUCTION

The steroid hormone testosterone is one of the main hormones involved in the modulation of social and sexual behaviour. For example, studies in a variety of taxa have shown that castration reduces sexual behaviour in males and that subsequent administration of testosterone generally restores it [1,2]. Likewise, testosterone levels are often elevated during periods of intense competition for mates and/or territories (e.g. [3–5]), suggesting that this hormone promotes male–male competition. However, this is only one side of the coin, as behaviour can also feed back on hormone levels on a short-term and a long-term basis [6]. Androgens are, for example, elevated after competition in humans [7] and after territorial fights in cichlid fish [8] or Siberian hamsters (*Phodopus sungorus*) [9] (for a review on vertebrates see [10]). Whereas many of these studies were done in captivity, the relationship between competitive behaviour and testosterone has also been studied extensively in the field, mostly by testing territorial male songbirds with a simulated intruder into their territories—so-called simulated territorial intrusion (STI) experiments. These studies were based on the 'challenge hypothesis' [5], which argues that males of monogamous biparental bird species should show an elevation in testosterone only during male–male conflicts, but maintain lower breeding baseline levels of testosterone during other times. These brief elevations in testosterone may promote a male's reproductive success by enhancing territorial and sexual behaviours, but prolonged high levels of testosterone should be avoided as they may interfere with paternal behaviour [11,12] and may incur other costs [13]. Thus, testosterone is thought to balance the trade-off between investment in territory defence and paternal care, and should only increase during times of social instability (i.e. when challenged by other males). As competitive interactions between males usually occur during territory establishment in early spring, these surges in testosterone have been considered to result in breeding season testosterone profiles typical for many temperate zone species; namely high testosterone levels (close to the physiological maximum, referred to as 'level C') at the beginning of the breeding season and lower testosterone levels during incubation and feeding of young. However, the experimental evidence for the challenge hypothesis has been mixed. While males of some songbird species respond with an increase in testosterone above breeding baseline levels after an STI (e.g. [14–16]), many others do not (e.g. [17–20]; reviewed in [21,22]). This difference

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between species may be related to differences in their life history. So far, three ecological hypotheses—the essential paternal care hypothesis, the short season hypothesis and the number of broods hypothesis—have been put forward to explain between-species differences in androgen responsiveness to male–male interactions [22]. These hypotheses are not mutually exclusive and are all based on the general idea that biparental species living in environments with strong time or resource limits for breeding should not increase testosterone during STIs, because high levels of testosterone may interfere with paternal care. In general, populations with short breeding seasons breed relatively synchronously. As a consequence, mating opportunities after egg-laying are rare, and males may maximize fitness by allocating most of their time and energy to paternal care of their current clutch rather than investing in male competition or extra-pair matings. This framework emerged from studies in arctic birds that have a very short and highly synchronized breeding season, and that do not modulate testosterone during STIs [23] ('short season hypothesis'). Building upon this short season hypothesis for arctic birds, Landys *et al.* [18] and Goymann *et al.* [21] observed in a comparative study that males of temperate zone species that raise only one brood per season also did not increase testosterone during STIs, whereas males of multiple-brooded species did ('number of broods hypothesis'). Goymann [22] generalized the short season hypothesis (originally proposed for arctic birds) and showed that the likelihood that males show a rise in testosterone during STIs increases with the length of the breeding season (i.e. males of species with a short breeding season do not increase testosterone during STIs, whereas males of species with a long breeding season show this androgen responsiveness to male–male interactions).

Life-history differences between species may not only translate into distinct hormonal responses to STIs, but may be related to further endocrinological differences in how individuals deal with social cues. Of particular importance is the idea that males of some species may not show an increase in testosterone during STIs because their levels of testosterone are already maximal [21,22]. This may be either because they are frequently challenged by other males or because they are physiologically constrained to further increase testosterone. This can be tested by injecting birds with gonadotropin-releasing hormone (GnRH). GnRH induces the pituitary to release luteinizing hormone (LH), which then stimulates the testes to secrete testosterone. Not many studies have tested the androgen response to GnRH in songbirds, but it is already evident that there are differences between species [24–29]. To the best of our knowledge, no study has yet tested the androgen response to STIs and GnRH in the same individual. This is, however, of paramount importance to find out whether individuals who do not mount an androgen response to male–male interactions would be capable of mounting such a response at all. If not, their testosterone levels may have been already maximal and they would not be able to respond to any social cues, including interactions between males or with receptive females.

Accordingly, in this study, we first tested the short season and the number of broods hypotheses by comparing populations of the same species that differ in the length of their breeding season and in the number of clutches. Following the predictions of the two hypotheses, we expected that the population with a short breeding season would not show an increase in testosterone during STIs, whereas the population with the long breeding season should do so. Second, by injecting GnRH after the STI, we tested whether testosterone does not increase during STIs because testosterone levels were already maximally elevated. We conducted STI experiments and GnRH challenges during territory establishment (when male–male interactions occur frequently and testosterone levels are expected to be high) and during the parental phase of the first clutch (when interactions between males are less frequent and testosterone levels are expected to be lower). This approach allowed us to differentiate whether the hypothalamic-pituitary-gonadal (HPG) axis of the tested males is not sensitive to territorial challenges at all and whether they therefore do not increase testosterone during any breeding stage or sensitivity changes during the breeding season.

Our study species was the black redstart (*Phoenicurus ochruros*), a highly territorial, socially monogamous songbird that covers a large breeding range from high to low altitudes within Europe [30]. High-altitude populations have a short breeding season with typically one brood, whereas low-altitude populations have a long breeding season and raise two or more broods [30]. To test whether different life histories led to distinct androgen responses to male–male interactions and to differences in HPG axis physiology in general, we challenged males of a low- and a high-altitude population with STIs and injections with GnRH during two breeding stages.

Black redstarts show a resurgence of territorial behaviour in autumn after moult, before they leave their breeding grounds. This behaviour offered an additional opportunity to assess seasonal changes in androgen responsiveness to STIs and GnRH. Therefore, we also collected data in autumn for the low-altitude population and compared the intensity of territorial behaviour and androgen responsiveness between the breeding season and autumn.

2. METHODS

Low-altitude male black redstarts were caught in 2008 between 1 and 30 April (territory establishment), between 19 May and 12 June (feeding 1st clutch) and between 19 September and 6 October (autumn territoriality) in Upper Barbaria (47° N, 11° E; 500–600 m above sea level). High-altitude male black redstarts were caught in the Southern Tyrolean Alps (46° N, 11° E; 1800–2500 m above sea level) between 5 and 15 May (territory establishment) and again between 18 June and 4 July (feeding young). Overall, we collected samples from 107 birds that were subjected to different experimental treatments.

(a) *Simulated territorial intrusion*

To elicit a territorial response, we placed a stuffed decoy into the centre of a territory and played back black redstart song using five different playbacks in random order (wav files, each repeated at a rate of eight strophes per minute). This procedure has been successfully used to elicit a territorial response in male black redstarts in another study [31]. As decoys, we used three different stuffed males in full adult

plumage that were protected by an inconspicuous cage made of a wire frame and a mist net. We recorded the following behaviours of the territory owner for 10 min: (i) latency to respond to the STI either by singing or approaching the decoy; (ii) the first time the male was in a 5 m radius around the decoy; (iii) the time the male spent in this 5 m radius; (iv) the time the territory owner was fluffed; and (v) the number of head noddings, which are typical threat postures of male black redstarts [31]. Furthermore, we noted whether the male attacked the decoy or sang at any time during the STI. After 10 min, mealworm-baited ground traps and spring traps were opened and the playback continued until the bird was caught (mean $\pm$ s.d.: 35 $\pm$ 20 min, range: 13.5–94 min).

Control males were caught either passively while searching for food or similar to STI males, with playback and presentation of a decoy within 10 min of the onset of the STI, and with the traps open from the beginning (mean $\pm$ s.d.: 246 $\pm$ 129 s, range: 60–480 s), following Wingfield & Wada [14], who demonstrated that an increase in testosterone during an STI can be observed only after 10 min of stimulation.

Immediately upon capture, a blood sample (approx. 120 µl) was taken after venipuncture from the wing vein (178 $\pm$ 112 s) and collected into heparinized capillaries. The bird was then injected with 50 µl chicken GnRH-I (Bachem H 3106; 1.25 µg dissolved in 50 µl isotonic saline; see [26]) into the pectoralis major muscle and kept in a holding bag. Another blood sample (approx. 120 µl) was taken after 30 min following procedures described by Moore *et al.* [27] and Wingfield *et al.* [29]. Control and experimental groups did not differ significantly in body mass ($t = 1.1$, d.f. = 67, $p = 0.3$), length of the right tarsus ($t = 1.2$, d.f. = 67, $p = 0.2$), length of the right wing ($t = 1.5$, d.f. = 68, $p = 0.1$) and cloacal protuberance (CP) volume (estimated by expressing it as a cylindrical shape: $V = \pi \times (\text{CP width}/2)^2 \times \text{CP height}$; $t = -1.8$, d.f. = 67, $p = 0.08$; see electronic supplementary material for detailed results on morphological measurements). In addition, each male was banded with a numbered aluminium ring (Vogelwarte Radolfzell) and a combination of two colour rings.

(b) *Plasma separation and hormone analysis*

Plasma was immediately separated by centrifugation with a Compur Minicentrifuge (Bayer Diagnostics). The amount of plasma was measured with a Hamilton syringe and stored in 500 µl ethanol [32]. After returning from the field, samples were stored at -80°C. Testosterone concentration was determined by direct radioimmunoassay (RIA) following Goymann *et al.* [33]. For details of the extraction, see the electronic supplementary material. Mean $\pm$ s.d. efficiency of the extraction with dichloromethane was 96 $\pm$ 9 per cent. The lower limit of detection of the assay was determined as the first value outside the 95 per cent confidence interval (CI) for the zero standard (B_{max}) and was 6.7 pg tube^{-1}. Samples were measured in duplicates in two assays, each containing samples from both populations. The intra-assay coefficients of variation were 4.7 and 4.2 per cent, respectively. The inter-assay variation was 5.5 per cent. As the testosterone antibody shows significant cross-reactions with 5a-dihydrotestosterone (44%), our measurements may include a minor fraction of this additional androgen.

(c) *Statistical analysis*

Data analysis was done with R v. 2.9.1 [34]. We used general linear models to analyse the influence of altitude and age (yearling or adult) on body mass, wing length, tarsus length and CP (electronic supplementary material). Behavioural data were first analysed including data from the low- and high-altitude populations without data from autumn. Second, we analysed behavioural data including only the low-altitude population to compare breeding season with autumn data. Behavioural data also include data from individuals that we were not able to catch within 2 h. We analysed whether the latency to first approach within 5 m (seconds), the time spent within 5 m (seconds) and agitation-related behaviours (head nodding, feather fluffing) differed between altitudes and life-cycle stages. We set territory establishment *a priori* as reference level in all models. Head nodding (factor loading: 0.70) and feather fluffing (factor loading: 0.70) were combined to one variable by using principle component analysis. The first principle component factor explained 60 per cent of the total variance and we used it as a score for agitation (agitation score).

Log-transformed post-capture testosterone levels, log-transformed GnRH-induced testosterone levels, and the absolute and relative (electronic supplementary material) increase from post-capture to GnRH-induced testosterone levels were analysed with general linear models for effects of treatment, altitude, breeding stage and age. We calculated combined models for the low- and high-altitude populations excluding low-altitude autumn data. In addition, we calculated models with data from the low-altitude population only, including testosterone data from the breeding season and autumn period. Furthermore, we used general linear models to check whether post-capture testosterone levels, GnRH-induced testosterone levels and the absolute increase in testosterone of STI males after GnRH injection were correlated with any of the behaviours shown during the STI. Not all males captured during an STI were injected with GnRH, resulting in different sample sizes for the various analyses.

To estimate androgen responsiveness to the STI and GnRH, we calculated effect sizes (Cohen's *d*; see [21,22]) using the program ESCI [35]. Significance was accepted at $\alpha < 0.05$ (two-tailed) and data are presented as (back-transformed) means and 95 per cent CIs.

3. RESULTS

(a) *Behaviour*

The STIs elicited similar behavioural responses as intrusions by real males or territorial conflicts with neighbours ([36]; B. Apfelbeck 2008–2010, personal observations).

(b) *Low-altitude population in Upper Bavaria*

Males responded to an STI similarly across life-cycle stages. Only the first approach within 5 m differed significantly between stages ($F_{2,56} = 5.0$, $p = 0.01$; table 1); males approached the decoy faster during territory establishment than when they were feeding the young of their first brood ($t = 2.61$, d.f. = 56, $p = 0.012$; table 1). But they approached the decoy as fast in autumn as during territory establishment ($t = -0.396$, d.f. = 56, $p = 0.69$; table 1). They spent similar amounts of time within 5 m of the decoy during all life-cycle stages ($F_{2,56} = 0.8$, $p = 0.4$; table 2). Likewise, the agitation score did not differ between territory establishment and the parental phase ($t = -0.54$, d.f. = 39, $p = 0.6$; table 1; because of differences in recording techniques, autumn agitation-related behaviours were not included). Also, the number

Table 1. Mean (±95% CI) intensity of behaviours measured during the STIs in relation to altitude, breeding stage and season.

	low altitude			high altitude	
	territory establishment $n = 31$	parental phase $n = 11$	autumn $n = 17$	territory establishment $n = 13$	parental phase $n = 25$
approach (s)	186 ± 45	335 ± 77	241 ± 75	263 ± 104	262 ± 74
time within 5 m (s)	317 ± 54	251 ± 80	285 ± 78	212 ± 89	207 ± 65
fluffing (%)	70 ± 10	76 ± 14		81 ± 14	60 ± 13
head nods per minute	2.9 ± 0.6	3.2 ± 1.7		3.7 ± 1.1	2.7 ± 1

Table 2. Results of the general linear models for post-capture and GnRH-induced testosterone levels, and the increase in testosterone caused by GnRH with respect to behavioural measurements during the STIs. Significant effects are printed in bold.

	post-capture testosterone		GnRH-induced testosterone		testosterone increase	
	test statistic	*p*-value	test statistic	*p*-value	test statistic	*p*-value
approach	$F_{1,37} = 1.3$	0.3	$F_{1,25} = 0.3$	0.6	$F_{1,24} = 2.18$	0.1
time within 5 m	$F_{1,37} = 0.7$	0.4	$F_{1,25} = 0.7$	0.4	$F_{1,24} = 1.6$	0.2
agitation	$F_{1,37} = 0.3$	0.6	$F_{1,25} = 0.7$	0.4	$F_{1,24} = 0.2$	0.6
attacks (yes, no)	$F_{1,37} = 0.04$	0.9	$F_{1,25} = 0.4$	0.5	$F_{1,24} = 0.3$	0.6
song (yes, no)	$F_{1,37} = 2.2$	0.1	$F_{1,25} = 0.05$	0.8	$F_{1,24} = 0.01$	0.9
STI duration	$\mathbf{F_{1,39} = 9.7}$	**0.003**	$F_{1,27} = 0.6$	0.4	$F_{1,26} = 0.2$	0.7
attacks (yes, no)	$F_{1,39} = 0.8$	0.4	$F_{1,27} = 1.4$	0.24	$F_{1,26} = 0.02$	0.9
STI duration × attacks	$\mathbf{F_{1,39} = 5.5}$	**0.01**	$F_{1,27} = 1.5$	0.23	$\mathbf{F_{1,26} = 5.6}$	**0.02**

of individuals that attacked the decoy did not differ between life-cycle stages (first 10 min of STI: Fisher's test, $p = 0.7$; STI until capture: Fisher's test, $p = 0.9$). However, fewer individuals sang during the STI in autumn than during the STIs during breeding (first 10 min of STI: Fisher's test, $p = 0.0003$; STI until capture: Fisher's test, $p = 0.007$).

(c) *High-altitude population in Southern Tyrol*

Males approached the decoy as fast and stayed as long within 5 m of the decoy during the parental phase as during territory establishment (approach: $F_{1,36} = 0.004$, $p = 0.9$; time within 5 m: Mann–Whitney *U*-test, $U = 159.5$, $p = 0.9$; table 1). However, males were more agitated during the STI in the territory establishment phase than during the parental phase ($F_{1,36} = 6.3$, $p = 0.02$; table 1).

(d) *Comparison of the low-altitude and high-altitude populations*

The latency to approach within 5 m of the decoy did not differ between breeding stages ($F_{1,76} = 3.1$, $p = 0.08$) and altitude ($F_{1,76} = 0.5$, $p = 0.5$; stage × altitude: $F_{1,76} = 3.5$, $p = 0.07$). Males of the high-altitude population spent slightly less time within 5 m than males of the low-altitude population ($F_{1,77} = 4.1$, $p = 0.05$). But there was no difference between breeding stages ($F_{1,77} = 3.5$, $p = 0.07$). Males from the high-altitude population were less agitated during the STIs in the parental phase than in the territory establishment phase (stage × altitude: $F_{1,76} = 4.2$, $p = 0.04$; breeding stage: $F_{1,76} = 2.2$, $p = 0.1$; altitude: $F_{1,76}$, $p = 0.8$).

(e) *Hormones*

(i) *Hormonal response to simulated territorial intrusions and gonadotropin-releasing hormone with respect to altitude*

Male black redstarts did not increase plasma testosterone in response to an STI ($F_{1,65} = 0.04$, $p = 0.84$; figure 1) regardless of altitude ($F_{1,65} = 0.14$, $p = 0.71$; figure 1) and breeding stage ($F_{1,65} = 0.09$, $p = 0.76$). This means their androgen responsiveness to male–male interactions was very low (mean (±95% CI) effect sizes: overall: $d = -0.07$ (-0.54; 0.4); low-altitude population: $d = -0.18$ (-0.79; 0.43); high-altitude population: $d = 0.03$ (-0.7; 0.75)). In a small subset of the STI experiments, the STIs induced real intrusions by neighbouring or other males. Post-capture testosterone levels of males facing a real intruder also did not significantly differ from those of controls or STI-only males ($F_{2,73} = 0.03$, $p = 0.9$; figure 2). GnRH-induced testosterone levels were significantly higher than testosterone levels before the injection (paired *t*-test: $t = -9.72$, $p < 0.0001$, $d = 1.1$ (0.8; 1.4); figures 1 and 3). Furthermore, testosterone levels before and after the GnRH injection were significantly positively correlated ($r = 0.61$, $p < 0.0001$; figure 4). The GnRH-induced increase in testosterone did not differ between control and STI-challenged males ($F_{1,66} = 1.6$, $p = 0.2$, $d = 0.36$ (-0.11; 0.83)). Thus, despite the fact that male black redstarts did not increase testosterone during STIs, they would have had the potential to do so because they were not physiologically limited. Post-capture testosterone levels did not differ between breeding stages ($F_{1,65} = 0.7$, $p = 0.41$, $d = 0.23$ (-0.24; 0.7); figure 3) nor between altitudes ($F_{1,65} = 0.2$, $p = 0.66$, $d = -0.14$ (-0.62; 0.33);

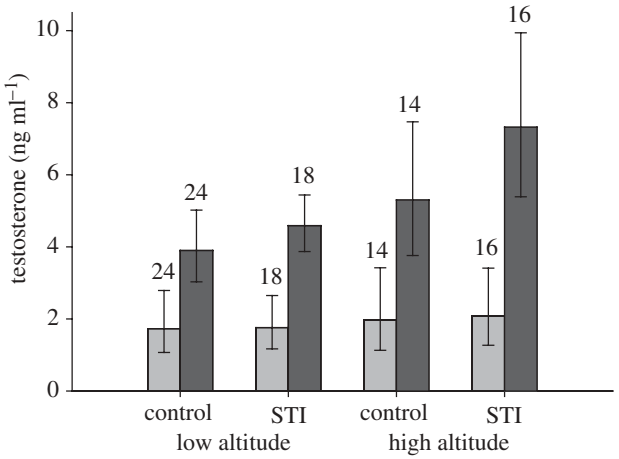

Figure 1. Back-transformed means (±95% CI) of post-capture testosterone levels did not differ between control and simulated territorial intrusion (STI) males of the low- and the high-altitude populations. However, testosterone levels increased after a GnRH injection within the same individual. Sample sizes are given above bars. Light grey bars, post-capture; dark grey bars, GnRH induced.

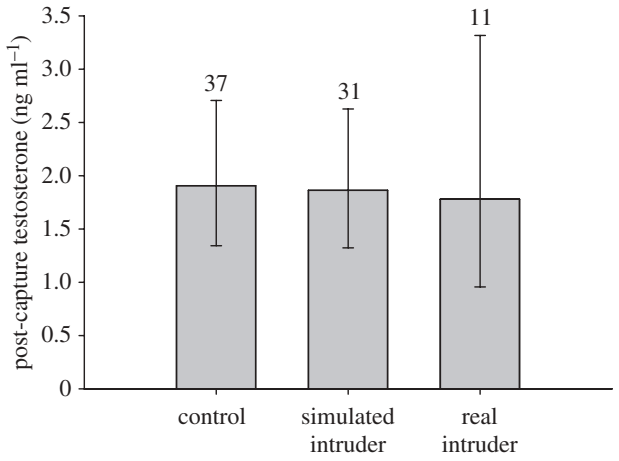

Figure 2. Back-transformed means (±95% CI) of post-capture testosterone levels did not differ between control males and males who were challenged with a simulated intruder or a real intruder. Sample sizes are given above bars.

figure 3). However, the physiological potential to release androgens was significantly higher during the territory establishment phase than during the parental phase ($F_{1,68} = 9.7$, $p = 0.003$, $d = 0.74$ (0.25; 1.22); figure 3). Correspondingly, the increase in testosterone was higher during territory establishment than during the parental phase ($F_{1,66} = 6.4$, $p = 0.01$, $d = 0.58$ (0.1; 1.05)). Furthermore, GnRH-induced testosterone levels were significantly lower in males from the low-altitude than from the high-altitude population ($F_{1,68} = 9.9$, $p = 0.002$, $d = -0.78$ (−1.7; −0.28); figures 1 and 3). Thus, the increase in testosterone was also significantly lower in males of the low-altitude than of the high-altitude population ($F_{1,66} = 6.7$, $p = 0.01$, $d = -0.71$ (−1.2; −0.22)). Between age classes there was no significant difference in post-capture testosterone levels ($F_{1,65} = 3.0$, $p = 0.08$), GnRH-induced testosterone levels ($F_{1,68} = 3.0$, $p = 0.088$) nor the increase in testosterone ($F_{1,66} = 0.63$, $p = 0.4$).

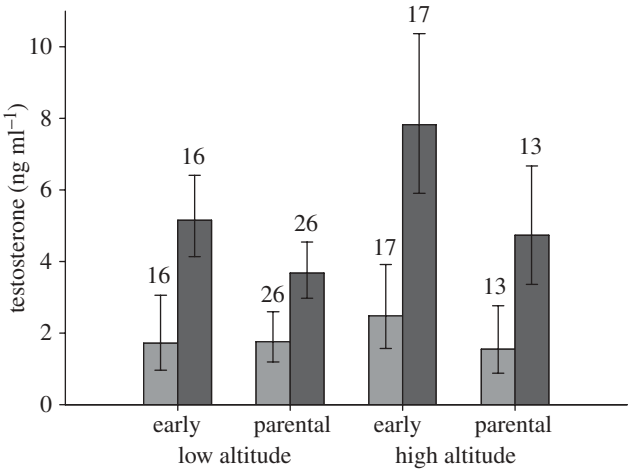

Figure 3. Back-transformed means (±95% CI) of post-capture testosterone levels were similar during the early and the parental breeding phase, and between the low- and the high-altitude population. However, the physiological potential to mount an androgen response to GnRH was significantly higher during the early breeding than during the parental phase, and higher in the high than in the low-altitude population. Sample sizes are given above the bars. Light grey bars, post-capture; dark grey bars, GnRH induced.

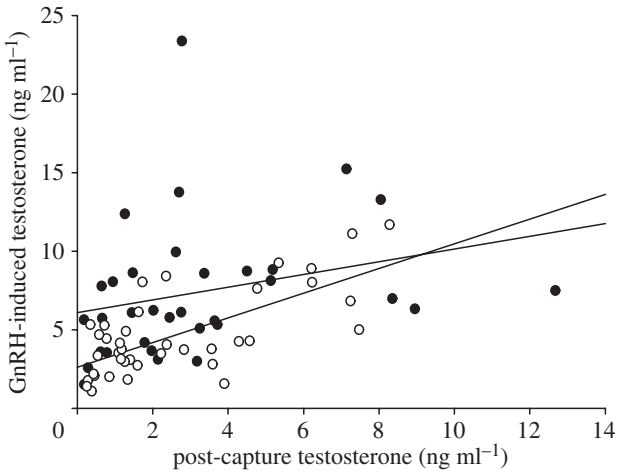

Figure 4. Post-capture testosterone levels were highly correlated with GnRH-induced levels ($r = 0.61$, $p < 0.0001$). Data are shown for both populations taken together for the early (black circles) and the parental breeding phase (white circles).

(ii) *Hormonal response to simulated territorial intrusions and gonadotropin-releasing hormone for the low-altitude population in relation to life-cycle stages*

Post-capture testosterone levels significantly differed with life-cycle stage ($F_{2,49} = 73.3$, $p < 0.0001$). *A priori* contrasts revealed that they did not differ between territory establishment and the parental phase ($t = 0.005$, d.f. = 49, $p = 0.99$, $d = 0.06$ (−0.56; 0.68)), but were significantly higher during territory establishment than in autumn (mean ±95% CI: 40 ± 8 pg ml^{-1}; $t = -6.73$, d.f. = 49, $p < 0.0001$, $d = 1.4$ (0.6; 2.2)). Testosterone levels did not increase during the STIs ($F_{1,49} = 1.6$, $p = 0.2$), and this was consistent between life-cycle stages (non-significant interaction: $F_{2,49} = 0.07$, $p = 0.9$).

GnRH-induced testosterone levels also differed with life-cycle stage ($F_{2,52} = 482$, $p < 0.0001$). They were

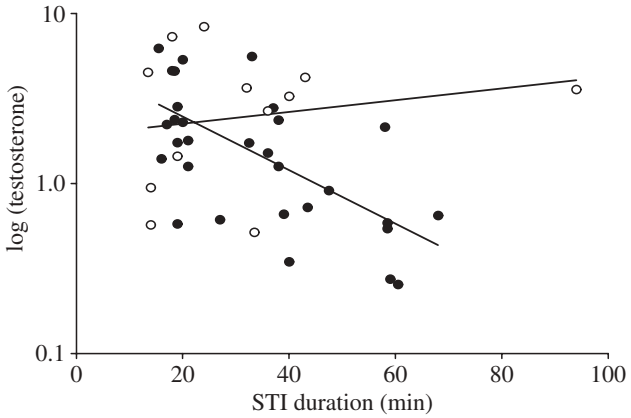

Figure 5. Post-capture testosterone levels plotted against the duration of the simulated territorial intrusion (STI). In males that attacked the decoy (white circles, $n = 12$), testosterone levels stayed high, while in males that did not attack the decoy, testosterone levels declined with STI duration (black circles, $n = 31$).

significantly higher during territory establishment than during the parental phase ($t = -2.2$, d.f. $= 52$, $p = 0.03$, $d = 0.85$ (0.4; 1.29); figure 3) and autumn (mean $\pm$95% CI: 41 ± 7 pg ml^{-1}; $t = -27.5$, d.f. $= 52$, $p < 0.0001$, $d = 3.7$ (2.4; 4.9)).

Likewise, the increase in testosterone caused by GnRH depended on life-cycle stage ($F_{2,49} = 7.0$, $p = 0.002$). The increase was higher during territory establishment than in autumn ($t = -2.4$, d.f. $= 49$, $p = 0.02$, $d = 1.4$ (0.6; 2.3)), but did not differ between territory establishment and the parental phase ($t = -1.5$, d.f. $= 49$, $p = 0.1$). The increase did not differ between control and STI males ($F_{1,49} = 0.54$, $p = 0.47$), and this was independent of life-cycle stage ($F_{2,49} = 0.15$, $p = 0.9$).

(iii) *Testosterone and behaviour*

Post-capture and GnRH-induced testosterone levels and the increase in testosterone caused by GnRH did not covary with the behaviours measured during the STIs (table 2). A linear model fitted for testosterone in relation to the duration of the territorial intrusion and to whether the territory owner attacked the decoy revealed that post-capture testosterone declined with the duration of the intrusion but remained high if males attacked the decoy (table 2 and figure 5). This result remains robust even when removing the 'outlier' in the upper right corner of figure 5 (STI duration: $F_{1,38} = 9.7$, $p = 0.003$; attacks: $F_{1,38} = 0.7$, $p = 0.4$; interaction: $F_{1,38} = 5.5$, $p = 0.02$). GnRH-induced testosterone levels did not show a significant relationship with STI duration, attacks nor the interaction between the two (table 2). In accordance with this, STI duration was positively correlated with the GnRH-induced testosterone increase in non-attacking males but not in males that attacked the decoy (table 2).

4. DISCUSSION

In contrast to our prediction, the androgen responsiveness of male black redstarts to an STI ($R_{\text{male-male}}$ *sensu* [21]) did not depend on the number of broods or the length of the breeding season: both populations studied did not increase testosterone when challenged with STIs, neither during the early breeding season nor when they were feeding the young of their first brood. However, in both populations injection of GnRH led to an increase in testosterone levels in control and STI males, demonstrating that all males would have had the physiological potential ($R_{\text{potential}}$ *sensu* [21]) to further increase testosterone during STIs. To our knowledge, this is the first study that measured the androgen responsiveness to an STI and the physiological potential to release androgens in the same individuals. Thus, the black redstart is the first species for which it has been shown that a lacking increase in testosterone during STIs is not caused by a restriction in the potential to increase testosterone. Furthermore, males facing a real intruder showed behaviours similar to those of males experiencing a simulated intrusion (see also [36]) and also lacked the expected increase in testosterone, suggesting that the simulated intrusion mimicked a real territorial threat.

(a) *Testosterone in relation to simulated territorial intrusions and gonadotropin-releasing hormone*

Interspecific comparisons of the challenge hypothesis confirm the influence of mating system on seasonal testosterone profiles (R_{season} *sensu* [21]) in birds [21,37], and of sexual and parental behaviour on testosterone levels in vertebrates in general [10]. However, in a situational rather than a seasonal context, the predictions of the challenge hypothesis often do not hold [21]. A central prediction is that in socially monogamous bird species with biparental care, aggressive interactions between males lead to short-term increases in testosterone above breeding baseline levels. These surges in testosterone are expected to cause a seasonal peak in testosterone at the beginning of the breeding season, when competition between males is particularly high [5]. On this seasonal basis, our data are in line with the challenge hypothesis as testosterone levels in black redstarts were highest during territory establishment and before a second clutch was initiated. During incubation, testosterone levels were low (B. Apfelbeck & W. Goymann 2008–2009, unpublished data). However, in a situational rather than a seasonal context, the predictions of the challenge hypothesis do not hold in black redstarts. Aggressive encounters between males do not seem to be the direct cause of seasonal peaks in testosterone, because black redstarts did not elevate testosterone levels during simulated or real territorial intrusions. Thus, in this species, the seasonal androgen response (R_{season}) is no proxy for, and does not predict the androgen responsiveness to, male–male interactions ($R_{\text{male-male}}$) [21,22]. Because this was also the case in various other species, several hypotheses have been proposed to relate the lack of testosterone response following male–male interactions to life-history differences between species (see §1). We tested one set of these hypotheses: the short breeding season and number of broods hypotheses [22,23] in two populations of black redstarts. But we found no evidence for a differential testosterone response to STIs depending on the length of the breeding season and number of broods: males of both populations did not increase testosterone when challenged with an STI. For the high-altitude population, this conforms to the idea that species breeding in severe environments should not elevate testosterone

during male–male interactions in order to minimize interference with parental care. However, males of the low-altitude population would have been expected to elevate testosterone during male–male interactions, because their breeding season is long and they raise multiple clutches. Thus, the comparison of two populations of black redstarts is not consistent with the pattern predicted by the short season or number of broods hypotheses. Hence, other (so far unconsidered) factors may influence whether some species increase testosterone during territorial challenges, while others do not. In this context, the actual function of short-term modulations of testosterone after territorial conflicts may be important. Especially, studies in mammals and fish suggest that the androgen release after defence of the home territory may be crucial for the formation of the winner effect, and thus may promote territoriality [38,39]. Black redstarts of the low-altitude population defend territories with low testosterone levels in autumn (i.e. after having finished moult and before they migrate). When they return the following spring, they typically breed on these territories [40]. In autumn, they show no androgen response to territorial or GnRH challenges. Thus, in black redstarts residency effects might be decoupled from androgen release after territorial conflicts. This may be in contrast to some other temperate zone songbirds, such as the song sparrow, in which testosterone increases during male–male interactions during the breeding season [14]. However, the relationship between the winner effect, territoriality and short-term testosterone release has not been studied in songbirds yet.

Until now, it was unknown whether any of the bird species that did not increase testosterone during STIs had the capacity to further increase testosterone: if testosterone levels were already maximal, the lack of a testosterone response to the challenge would not be too surprising [22]. The black redstart is the first species for which we can now exclude this explanation: although males of both populations did not increase testosterone during STIs, both populations responded with a substantial increase in testosterone after injections with GnRH. Hence, males did not have maximum testosterone levels during any breeding stage and would have had the capacity to modulate circulating testosterone during a social challenge. Similarly, male rufous-collared sparrows (*Zonotrichia capensis*) and Gambel's white-crowned sparrows (*Zonotricha leucophrys gambelii*) also increase testosterone after a GnRH challenge [27], but do not modulate testosterone levels after a territorial dispute [41,42]. STI and GnRH challenges were, however, performed on different sets of individuals in these studies and thus could not exclude the possibility that testosterone levels were already maximal in STI-challenged individuals. These and our data indicate that, with respect to the effects of GnRH, species that do not increase testosterone when challenged with an intruder are similar to species that increase testosterone during STIs: the latter also respond to GnRH with an increase in testosterone [15,16,26,27].

In species that are responsive to GnRH, but not to male–male interactions, other social cues, such as sexual interactions with females [43,44], may modulate plasma testosterone levels [21,22]. Often it is difficult to distinguish whether the seasonal peak in testosterone coincides with the time period of frequent male–male interactions or the time period when females are most fertile, or both. Accordingly, in the low-altitude black redstart population, we observed a peak in testosterone during the parental phase of the first brood when many females had already started a second clutch (B. Apfelbeck 2008–2010, personal observations). During this time, overt male–male competition does not seem to be particularly strong, and any socially induced peak in testosterone may be more probably related to male–female interactions. In contrast to black redstarts, both male–male and male–female interactions may shape seasonal testosterone patterns in males of species that mount an androgen response both to STIs and GnRH. Song sparrows (*Melospiza melodia*), for example, show a peak in testosterone upon arrival at their breeding grounds when they establish territories and again when their mates start egg-laying [45].

If the amount of testosterone produced is relevant for the processing of social cues from other males or females, then the capability to do so probably changes during the life cycle. Similar to dark-eyed juncos [26], the capacity of male black redstarts to mount a testosterone response after a GnRH injection differed between life-cycle stages: GnRH-induced testosterone levels were significantly higher during the early breeding season than when they were feeding nestlings or fledglings (and during autumn, GnRH did not induce higher testosterone at all). In combination with studies that also measured LH release after GnRH injection [26,29], our data also suggest that the seasonal differences in testosterone release are, at least to some extent, regulated at the level of the testes. Possibly, the lower capacity to raise testosterone during the parental phase might be an adaptation to avoid interference of high testosterone levels with paternal care [5]. These data also demonstrate that seasonal peaks in testosterone do not necessarily reflect maximum physiological levels ('level C') as postulated by the challenge hypothesis [5], and that 'level C' is not fixed throughout the breeding season but changes depending on the breeding sub-stage.

Although testosterone levels before the GnRH injection were similar in black redstarts of the high- and the low-altitude populations, the increase after a GnRH injection was higher in males of the high-altitude population. Thus, the morphological differences between the two populations (electronic supplementary material) correspond with physiological differences in the regulation of the HPG axis. Similarly, Moore and colleagues [27] found the highest increase in testosterone after a GnRH injection in a high-altitude tropical species when comparing closely related *Zonotrichia* species in their physiological response to GnRH. Furthermore, this is in line with a comparative study in tropical birds demonstrating that seasonal androgen maxima are highest in species with short breeding seasons and at high altitudes [46]. Our study on black redstarts suggests that such patterns may also exist in temperate zone species and that such relationships may not only exist between species, but also within populations of the same species breeding at different altitudes.

(b) *Testosterone and behaviour*

GnRH-induced maximum testosterone levels were highly positively correlated with post-capture testosterone levels

and showed large inter-individual differences. Jawor *et al.* [26] found that in dark-eyed juncos, the androgen response to GnRH was repeatable within individuals and While *et al.* [47] found repeatable baseline testosterone levels in individuals of the Australian lizard (*Egernia whitii*). These findings raise the possibility that differences in baseline and/or maximum testosterone levels are related to individual differences in behaviour. However, individual correlations between testosterone and behaviour are rarely found (e.g. [48,49]). In our study, differences in post-capture and GnRH-induced testosterone levels were not related to differences in the behavioural response to a simulated intruder. Unexpectedly, post-capture testosterone levels declined with STI duration so that males that were caught at a later time point during the intrusion had lower testosterone levels than males caught earlier. However, this was not true for males that attacked the decoy: in these males, testosterone levels remained high. During STIs, territory owners cannot evict the intruder and, therefore, testosterone levels might start to decline during long-lasting intrusions [50] (but see [51]). Alternatively, during STIs, males are likely to be more active than during other periods, resulting in an increase in their metabolism: If the secretion of testosterone from the testes remains constant, but clearance increases owing to a higher blood flow through the liver, this may result in declining testosterone concentrations. But why did males that attacked the decoy maintain higher testosterone concentrations? Although there is little evidence for a relationship between coping style or personality and testosterone [52], a recent study of European stonechats by C. Muck & W. Goymann [53] showed that individuals that attacked their mirror image had higher baseline testosterone levels than individuals that did not attack the mirror. Thus, our observation in black redstarts is consistent with a situation in which individuals with a bold personality express higher levels of testosterone than individuals with a more shy personality, regardless of the situation.

5. CONCLUSIONS

Although differences in the androgen physiology of two black redstart populations exist, the fact that males of both populations do not mount an androgen response to STIs but to GnRH indicates that the regulation of territorial defence is similar in both populations. Thus, the length of the breeding season or the number of broods cannot universally explain between-species differences in androgen responsiveness to territorial challenges. Because black redstarts increased testosterone after injections with GnRH, they would have had the physiological capacity ($R_{potential}$) to increase testosterone during STIs. In other vertebrate taxa, short-term testosterone increases after male–male conflicts have been suggested to be responsible for the winner effect, and thereby reinforce territoriality. However, in species like the black redstart, which defend breeding territories in autumn, territory defence may be decoupled from short-term testosterone modulations. We suggest that the extent to which the winner effect and territoriality are coupled to short-term testosterone release might be a promising factor to explain the existing variation in androgen responsiveness to male–male interactions.

All experimental procedures were approved by the governmental authorities of Upper Bavaria and Southern Tyrol.

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Simulating winning in the wild — The behavioral and hormonal response of black redstarts to single and repeated territorial challenges of high and low intensity

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ABSTRACT

In many vertebrates testosterone increases during aggressive interactions and the surges in this hormone may be responsible for the winner effect. So far studies on this relationship have been done in captivity only, because simulating a winning situation for a territory owner in the field is difficult. However, an increasing number of studies show that territorial aggression is not necessarily accompanied by elevated testosterone after a single simulated territorial intrusion (STI) and therefore it has been proposed that STIs may even create a losing experience. We examined whether free-living male black redstarts (*Phoenicurus ochruros*) show changes in androgens, corticosterone and behavior following repeated STIs of high or low intensity and in contrast to being challenged only once. Repeated intrusions had no influence on androgen and corticosterone levels regardless of intrusion intensity. In contrast, the behavioral response changed over days depending on the intensity of the intrusion. Only birds challenged with high-level intruders approached the decoy significantly faster during the third intrusion than during the first one, stayed closer to the decoy, and sang more songs than males challenged with low-level intruders. Thus, although black redstarts reacted differently to STIs varying in frequency and intensity, these behavioral differences were not reflected in androgen or corticosterone levels. Our data show that it is unlikely that STIs induce a losing experience. Furthermore, they indicate that a hormonal effect of winning an encounter may not be universal in vertebrates and may depend on the ecological or life-history context.

Introduction

The sex steroid testosterone plays a critical role in the modulation of secondary sexual traits, territorial aggression, and courtship of male vertebrates (reviewed in Wingfield et al., 2006). In many seasonally reproducing vertebrates, testosterone levels change over the year and induce morphological, physiological and behavioral changes required for breeding (Lincoln et al., 1972; Moore, 1986; Wingfield and Farner, 1993). Superimposed on these seasonal changes in testosterone levels the challenge hypothesis predicts further brief increases in testosterone during periods of social instability, i.e. during territorial conflicts or during mate competition (Wingfield et al., 1990). Originally developed for birds, the predictions of the challenge hypothesis gained large support from data based on seasonal testosterone profiles in birds and other vertebrates (R_{season} sensu Goymann et al., 2007a, see also Hirschenhauser et al., 2003; Hirschenhauser and Oliveira, 2006). However, when actual changes in testosterone concentrations during social interactions are investigated, the support for the challenge hypothesis becomes equivocal. On the one hand a large number of studies demonstrated rapid effects of social interactions on plasma concentrations of testosterone in a variety of vertebrate taxa (e.g. Archer, 2006; Hirschenhauser et al., 2004; Oyegbile and Marler, 2005; Wingfield and Wada, 1989). On the other hand, however, a recent comparison within birds has revealed that most species studied to date did not show the expected increase in testosterone after simulated territorial intrusions (STI, $R_{male-male}$ sensu Goymann et al., 2007a; Goymann, 2009).

Also, the function of short-term increases in testosterone after aggressive interactions is still barely understood. One idea is that testosterone mediates the winner effect (reviewed in Oliveira, 2004; see also Oyegbile and Marler, 2005; Oliveira et al., 2009). According to the winner effect an individual is more likely to win future aggressive encounters after winning previous encounters independent of intrinsic contest skills (Bergman et al., 2010; Chase et al., 1994; Oliveira et al., 2009; Oyegbile and Marler, 2006; Trainor et al., 2004). In vertebrates, winning an encounter is typically accompanied by a short-term increase in testosterone, while losing a contest is followed by a decrease in this androgen. However, as mentioned above, many species do not show an increase in testosterone during aggressive encounters. In some of these species repeated winning may be required to induce a significant rise in testosterone (Oyegbile and Marler, 2005).

Also, some authors have suggested that the lack of an increase in testosterone during some STI studies may be due to the induction of a

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loser effect (losing a contest tends to decrease the probability of winning in future contests, Hsu and Wolf, 1999): natural territorial encounters may be brief and typically the territory owner evicts the intruder within a few minutes. In contrast, during a STI the territory owner is unable to expel the intruder from the territory for a prolonged period of time. This may be perceived by the territory owner as if it was losing the encounter and lead to a decrease in testosterone levels (Goymann et al., 2007a; Goymann, 2009; Kempenaers et al., 2008).

A second important group of hormones involved in winning or losing a fight are glucocorticoids such as corticosterone. Short-term elevations in corticosterone levels during male–male encounters have been shown to promote aggression (Mikics et al., 2004; Øverli et al., 2002; Summers et al., 2005; Woodley et al., 2000). But in some species losers of a fight need longer to return to baseline levels than winners (Øverli et al., 2000). Prolonged elevated corticosterone levels have been associated with decreased aggression, submission and losing male–male interactions (Nock and Leshner, 1976; Øverli et al., 2002; Sloman et al., 2001; Schuett and Grober, 2000; Summers et al., 2005). Therefore, these hormones may be used as indicators of how an individual perceives a social challenge.

From the above follows that the challenge hypothesis and the winner and loser effect are related phenomena, as short-term increases in testosterone during or after male–male interactions ("challenge hypothesis") may act as physiological mediators for winning also in future encounters ('winner effect'; Oliveira, 2004; Oliveira et al., 2009; Oyegbile and Marler, 2005). Studies testing this "winner–challenge"-hypothesis are, however, still rare and have been conducted under laboratory conditions only (Oyegbile and Marler, 2005; Oliveira et al., 2009; Oliveira et al., 2002; Gleason et al., 2009). The aim of the present study was to investigate two factors related to the winner effect that may explain the lack of an increase in testosterone during STIs in male black redstarts (*Phoenicurus ochruros*) in the field. Black redstarts, like many other birds (reviewed in Goymann et al., 2007a; Goymann, 2009), violate predictions of the challenge hypothesis, i.e. they react aggressively to STIs, but they do not increase testosterone in response to this challenge (Apfelbeck and Goymann, 2011). In this previous work we have demonstrated that despite the lack of a direct testosterone response to STIs black redstarts would have the physiological capacity to raise testosterone: males that did not show an increase in testosterone during STIs showed a pronounced increase in testosterone when injected with gonadotropin releasing hormone (GnRH; Apfelbeck and Goymann, 2011). Here, we tested if brief and repeated STIs with a vanishing intruder influence future behavior and hormone levels. We either confronted free-living male black redstarts with a single STI or with repeated STIs with intruders that automatically disappeared after 10 min. These intrusions were conducted either with low or high intensity, thus simulating a weak or a strong intruder. With this paradigm we attempted to simulate several winning experiences in the field before capturing the focal male. This experimental setup does not allow us to directly test the winner effect, i.e. a higher probability of winning an encounter after prior winning experiences. Also, we could not directly test for a loser effect, i.e. a higher probability of losing an encounter after having lost previous encounters. However, winners or losers of a contest normally show pronounced differences in behavior (and the winner or loser effect is often accompanied by distinct changes in behavior). Winners of a fight usually react faster and more aggressive in subsequent contests, while losers tend to become more passive (Hsu et al., 2006).

Thus, in this paper, we test three hypotheses that may explain the lack of a hormonal response of various species to single STIs: (1) Multiple (winning) territorial encounters may be needed to increase testosterone or (2) STIs may create a losing experience for territory holders, during which territorial birds do not increase testosterone. Finally, (3) the hormonal response may depend on intrusion intensity. We evaluate these hypotheses based on patterns of aggressive behavior that are indicative of winning and losing in other species (e.g. Hsu et al., 2006) and differences in androgens and corticosterone to single versus repeated STIs with low- or high-level intensity encounters.

If male black redstarts should require multiple territorial interactions before they show a rise in testosterone, we predict that redstarts caught after experiencing and "winning" three consecutive STIs before capture (during a final fourth STI) should express higher levels of testosterone than individuals caught after just one STI. With respect to behavior challenged territorial males should intensify their behavioral response over days and should react stronger towards the more threatening high-level intruders than towards the less threatening low-level intruders (hypothesis 1).

If, on the other hand, STIs induce a losing experience (hypothesis 2) we predict that redstarts will show a weaker territorial response (e.g. an increase in the latency to approach the decoy) during consecutive STIs, a decrease in testosterone levels and an increase in corticosterone levels.

However, producing a winner or a loser may depend on intrusion intensity (hypothesis 3). In this case, a low-level intruder should be less likely to induce a losing experience than a high-level intruder. Hence, a high-level intruder may be more likely to induce a losing experience and focal males may show a weak territorial response, no increase in testosterone and an increase in corticosterone. In contrast, a low-level intrusion should be less intimidating and more likely to induce a winning experience — leading to a strong territorial response, an increase in testosterone and lower levels of corticosterone.

Material and methods

Study species and study sites

We studied European black redstarts between April 9th and June 13th 2008 at two sites in Upper Bavaria: one south of the Ammersee (N 47°, E 11°) and the other one in the Chiemgau (N 47°, E 12°). Black redstarts are small socially monogamous songbirds, which are sexually dimorphic and dichromatic. Redstarts live in mostly open areas interspersed by one or several elevated structures, which are frequently used as singing posts (Landmann, 1996). The breeding season starts about end of April, when territories are fully established and continues until August, when redstarts begin to molt. During the study period, we grouped birds into three breeding sub stages: territory establishment, nest building and incubation, and feeding young. Plasma testosterone levels in male black redstarts follow a seasonal profile comparable to other socially monogamous and biparental songbird species: testosterone levels are highest at the beginning of the breeding season, slightly lower but still high during the parental phase of the first brood when they start preparing a second clutch and decline afterwards to very low levels during molt and in autumn (Apfelbeck & Goymann, unpublished data).

Experimental procedures

STIs are a commonly used method to investigate the behavioral and hormonal reaction of a focal territorial individual to an intruder into its territory (e.g. Goymann et al., 2008; Landys et al., 2007; Moore et al., 2004; Wingfield, 1985; Wingfield, 1994) and have been successfully used to induce a territorial response in male black redstarts previously (Apfelbeck and Goymann, 2011). Typically a live or stuffed decoy is employed in combination with playback of territorial songs.

For the experiment we used stuffed decoys protected by an inconspicuous cage made of a wire frame and a mist net mounted on a tripod. A string attached to the wire frame allowed to remotely remove the decoy: by pulling the string from a distance of about 30 m

the decoy disappeared into a darkgreen plastic cylinder. We put a remote-controlled loudspeaker (Foxpro Scorpion, digital game caller) underneath the decoy to play back the territorial song of a potential rival. To control for pseudoreplication we used five different playbacks and two different decoys in adult male plumage.

We observed the birds from about 20 to 30 m distance and recorded the following behaviors: (1) time between start of playback and first reaction (response latency), (2) first time approaching a range of 5 m to the decoy, (3) time spent within 5 m of the decoy, (4) number of attacks, (5) number of songs and (6) the frequency of head noddings which is linked with aggression or agitation. For the STI trials with repeated challenges we observed and recorded the behavior within the first 10 min of playback and also for the following 10 min after the decoy and playback had been removed. For the single trial STIs and the final STI of the repeated challenges we recorded the behavior for the first 10 min before opening the traps.

In the experiment, we used two kinds of STIs that varied in intensity (high and low) and frequency, thus resulting in four experimental groups. The high-level STI was a stuffed decoy that was moving up and down, thus simulating the 'head nodding' and 'tail flicking' behaviors that redstarts frequently show during territorial conflicts. This display was combined with playback of high song frequency with 8 songs played back per minute for 10 min. The low-level STI consisted of a static, non-moving decoy and playback of a low song frequency. Here, we played back 8 songs per minute for the first 2 min of the STI, another 8 songs per minute in the sixth minute of the 10 min interval, and no song for the rest of the 10 min period.

With regard to STI frequency we distinguished between two groups: the first group (repeated STI challenge) was challenged with different conspecific STIs three days in a row for 10 min using a different playback and randomly one of the two decoys (using every decoy twice with each focal male) every day. On day four we caught the birds after another 10 min STI using mealworm-baited ground traps and tree traps placed in the vicinity of the decoy and with continued playback of high or low intensity. The second group was caught after a single conspecific STI conducted in the same manner as in the group caught after repeated STI challenges (for both groups capture time: mean ± 95% CI: 30 ± 8 min, range 3–99 min). To additionally control for a potential disturbance due to setting up the STI equipment (as done during the repeated STIs) we initially "challenged" the birds of the single STI group with a heterospecific mount of a European robin (*Erithacus rubecula*) and with playback of robin song during the two consecutive days before the conspecific STI. However, redstarts completely ignored the heterospecific STIs and are used to the presence of humans, because they live within human settlements. We, thus, decided to catch most of the birds of the single STI group without conducting the heterospecific robin STI (6 redstarts challenged with a robin, 19 without previous robin challenges).

After capture we obtained two blood samples, one (~60 μl) within 3 min (mean 2.1 ± 0.4 min) to determine circulating corticosterone levels and a second one (~120 μl) within 5 min (mean 3.3 ± 1.0 min) to determine testosterone concentrations. For blood sampling, we punctured the left wing vein and collected the blood with heparinized capillaries. Blood samples were immediately centrifuged with a micro-centrifuge (Bayer Diagnostics). The amount of plasma was measured with a Hamilton syringe for later determination of the hormone concentration and stored in 500 μl ethanol to preserve steroid hormones above 0 °C (Goymann et al., 2007b). In five cases we were not able to collect a sample within 3 min for the corticosterone determination and thus took only the sample for testosterone. After returning to the lab (1–6 days) samples were stored at −80 °C until analysis.

The birds in all experimental groups did not significantly differ in body mass ($F_{3,31} = 0.3$, $p = 0.8$), tarsus length ($F_{3,31} = 0.9$, $p = 0.4$), wing length ($F_{3,31} = 0.7$, $p = 0.5$) and cloacal protuberance (CP) volume (estimated by expressing it as a cylindrical shape ($V = \pi *$ (CP width/2)^2*CP height; $F_{3,31} = 1.0$, $p = 0.4$). All experimental procedures were approved by the governmental authorities of Upper Bavaria.

Hormone assays

Testosterone and corticosterone were measured by radioimmunoassay following the procedure described in Apfelbeck and Goymann (2011) and Goymann et al. (2006). Extraction recovery (mean ± STD) was 95 ± 2% for testosterone and 96 ± 2% for corticosterone, respectively. The concentrations of all samples, including the standard curve, were calculated with Immunofit 3.0 (Beckmann Inc., Fullerton, CA, USA). The lower detection limit of the assay was 0.78 pg/ml for testosterone and 6.04 pg/ml for corticosterone. All samples were above the detection limit of the assays and were analyzed within one assay per hormone. The intra-assay coefficient of variation was 0.3% for testosterone and 3.6% for corticosterone; the intra-extraction coefficient of variation was 6.2% for testosterone and 12.0% for corticosterone. Because our testosterone antibody shows some cross-reaction with 5α-dihydrotestosterone (44%) our measurement may include a fraction of 5α-DHT, another potent androgen binding to the androgen receptor. We, therefore, refer to androgens instead of testosterone for the remainder of this contribution.

Statistical analysis

Data analysis was done with R version 2.9.1 (R Development Core Team, 2008).

Post-conflict androgen and corticosterone concentrations obtained after the single STI or the final STI of the repeatedly challenged males were log-transformed prior to analysis to ensure normal distribution and analyzed with general linear models. We started with a model including the experimental factors and their interaction (intruder type, number of challenges) and all further biologically relevant factors (androgens: breeding stage, STI duration; corticosterone: breeding stage, time between capture and blood sampling and STI duration). Experimental factors were always retained in all models. Interactions or other factors were removed successively if their p-value was greater than 0.1.

To test whether repeated STIs of different intensity influence future behavior of territorial males we analyzed the behavior of repeatedly challenged males including also those that we were not able to catch during the final STI. All behavioral variables except the number of songs (see below) were analyzed with linear mixed models including bird ID as a random factor, thereby accounting for repeated measures of the same individual. If necessary the dependent variables were log-transformed prior to analysis to account for normal distribution of the residuals. All initial models included the experimental factors and interactions (decoy present or not, day (1–3), intrusion type (high- or low-level), day*intruder and decoy*intruder). Day 4 was not included in the analysis of behavioral data because during this day the STI was continued until capture of the bird. Furthermore, we included capture date instead of breeding stage (as with this finer scale we reached better model fits) and whether the males were finally caught or not. Experimental factors were always retained in all models. Interactions or other factors were removed successively if the p-value was greater than 0.1.

The frequency of head nods (nods per min) was corrected for the time we did not see the focal male (response latency and time obscured by vegetation or other cover). The time spent in an area 5 m around the decoy was analyzed both as absolute time around the decoy and as percentage of the total time the bird was seen (duration of STI minus response latency and time when the bird was obscured by vegetation and we did not know exactly how far from the decoy it was). Both models produced similar results. However, the model fit was better when the relative time was used as dependent variable. Thus, only these results are shown. As the number of songs were

count data, we used generalized mixed models with Poisson distribution allowing for a random slope (day) and intercept (bird ID) and present the data as medians and their interquartile ranges.

Significance was accepted at $\alpha<0.05$ (two-tailed) and data are presented as (back-transformed) means and 95% confidence intervals (CI).

Results

Altogether we tested 54 male black redstarts and were able to catch 36 (67%) of them. Of these 36 caught birds 8 males experienced a single high-level STI and 8 males a single low-level STI. 10 birds were repeatedly challenged with high-level STIs and another 10 birds with repeated low-level STIs, thus keeping the experimental design balanced.

Behavior

Approach — first time within 5 m

Males that were confronted with repeated high-level STIs approached the area within 5 m of the decoy faster during each consecutive day, while there was no change over subsequent days in males tested with repeated low-level STIs (intruder type: $F_{1,25}=6.5$, $p=0.02$, day: $F_{2,42}=1.3$, $p=0.3$, interaction: $F_{2,42}=4.1$, $p=0.02$, Fig. 1). Thus, only with high-level STIs the approach latency differed between the initial and the third challenge. Date had no significant influence on approach latency, but there was a slight trend for territory owners taking longer to approach the decoy later in the season ($F_{1,42}=3.7$, $p=0.06$).

Time within 5 m

Males spent significantly more time in the area 5 m around the decoy during the first 10 min of all STIs, when the decoy and the playback were present, than during the 10 min after we had removed the decoy and playback ($F_{1,116}=11.5$, $p<0.001$, Fig. 2). This was more pronounced in males challenged with high-level STIs, which remained longer in the 5 m range than males challenged with low-level STIs ($F_{1,116}=4.9$, $p=0.03$, Fig. 2). The proportion of time spent within an area of 5 m around the decoy did not significantly change during subsequent challenges, but showed a tendency for territory owners spending more time close to the decoy on day three than on day one ($F_{2,116}=2.7$, $p=0.07$). Thus, all males clearly responded to the stimuli and the response did not change from the initial to the third challenge.

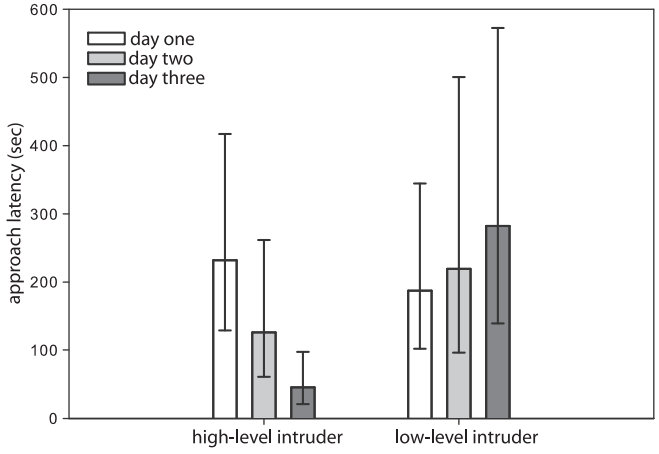

Fig. 1. Back-transformed means (±95% CI) of the approach latency. Males challenged with a high-level intruder (n = 13) approached the decoy significantly faster during the third than during the first STI. This was not the case in males challenged with a low-level intruder (n = 13).

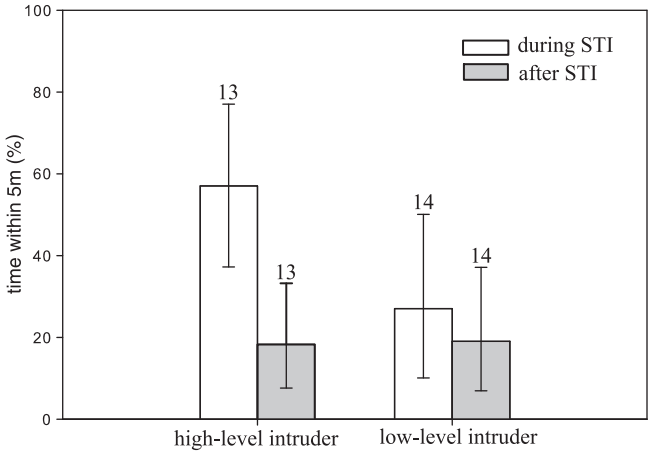

Fig. 2. Back-transformed means (±95% CI) of the relative amount of time that males spent within 5 m of the decoy. Males challenged with the high-level intruder stayed significantly longer close to the decoy during the STI than males challenged with the low-level intruder. Sample sizes are given above bars.

Agitation — head nodding per minute

Males were much more agitated during the STI than in the 10 min after removal of decoy and playback ($F_{1,115}=40.8$, $p<0.0001$). Neither type of intrusion ($F_{1,24}=0.9$, $p=0.4$) nor day of the experiment ($F_{2,115}=0.2$, $p=0.8$) had an effect on the number of head nods per minute. However, males that were finally caught were significantly more agitated during all STIs than males that were never caught ($F_{1,24}=6.1$, $p=0.02$).

Songs

Males sang significantly more songs during the 10 min after the STI than during the 10 min when the decoy and the playback were present ($z=-14.9$, $p<0.0001$, Fig. 3). In addition, they sang significantly more songs when confronted with high-level than with low-level intrusions ($z=-2.2$, $p=0.03$, Fig. 3). The number of songs sang did not differ significantly between consecutive STIs ($z=0.6$, $p=0.5$). Date had no significant effect on the number of songs sang,

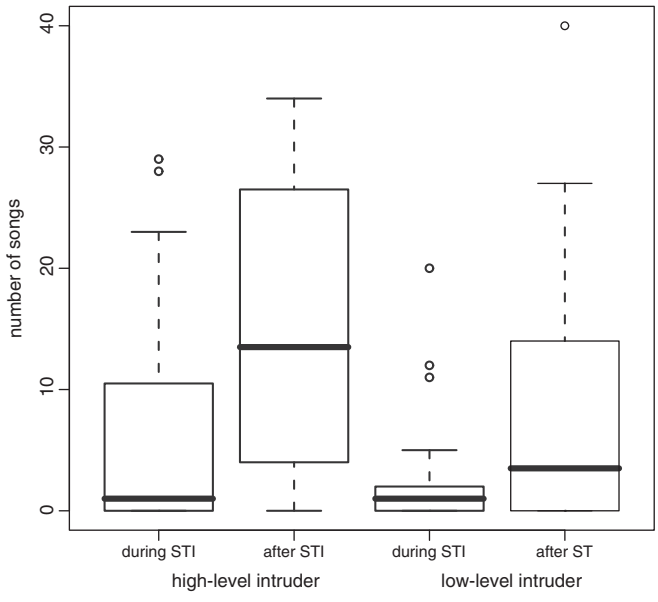

Fig. 3. Medians, interquartile ranges and 95% CIs of the number of songs males sang during and after the STI. Males challenged with the high-level intruder (n = 13) sang significantly more songs than males challenged with the low-level intruder (n = 14) during all STIs regardless of STI frequency.

but there was a trend that territory owners sang less later in the breeding season ($z = -1.9$, $p = 0.06$).

Hormones

Androgens

Post-conflict androgen levels were similar in males challenged with a single STI and those challenged with repeated STIs over the course of four days ($F_{1,31} = 0.2$, $p = 0.6$, Fig. 4). Furthermore, low- or high-level intrusion had no significant influence on post-conflict androgen levels ($F_{1,31} = 0.01$, $p = 0.9$, Fig. 4). However, androgen levels significantly varied with breeding stage ($F_{2,31} = 7.5$, $p = 0.002$). A priori set contrasts revealed that androgen levels of males were significantly lower when females were incubating eggs than during territory establishment ($t = -3.4$, $p = 0.002$). During parental care androgen levels were similar to levels during territory establishment ($t = -0.28$, $p = 0.8$), most likely because the pairs were close to initiating the second brood.

Corticosterone

Post-conflict corticosterone levels did not differ significantly between males that were challenged with conspecific STIs once or repeatedly over the course of four days ($F_{1,26} = 0.15$, $p = 0.7$, Fig. 5). Post-conflict corticosterone levels also did not differ significantly between males that were challenged with low- or high-level STIs ($F_{1,26} = 2.1$, $p = 0.2$, Fig. 5). However, post-conflict corticosterone concentrations significantly decreased with STI duration during the single and the final STI in males that were challenged with high-level STIs, but not in males that were confronted with low-level STIs ($F_{1,26} = 8.5$, $p = 0.007$, Fig. 6). Overall, post-conflict corticosterone levels during the single and the final STI were significantly positively correlated with testosterone levels ($r = 0.46$, $N = 29$, $p = 0.009$).

Neither androgen nor corticosterone levels showed any significant correlations with any of the behaviors measured (data not shown).

Discussion

We have previously shown that black redstarts violate the predictions of the challenge hypothesis, i.e. they are socially monogamous and males provide a substantial amount of parental care, but they do not increase testosterone levels during simulated territorial intrusions (Apfelbeck and Goymann, 2011). Here, we investigated three factors related to the winner effect that may have had the potential to explain the lack of an increase in androgens during STIs in this species.

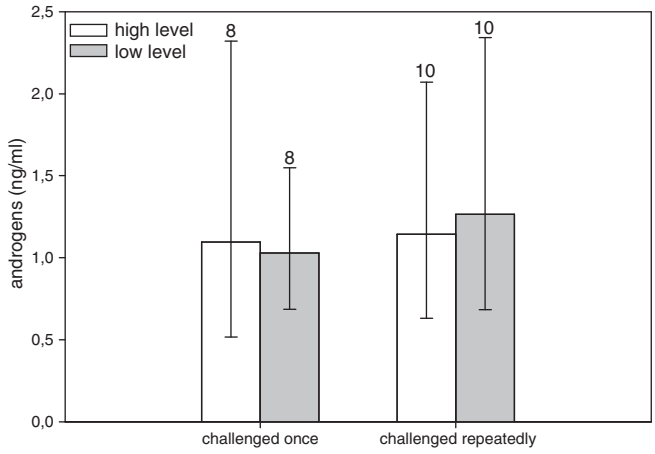

Fig. 4. Back-transformed means (±95% CI) of androgen levels did not differ between males that were challenged only once and males that were sampled after the final of the repeated challenges independent of intruder type. Samples sizes are given above bars.

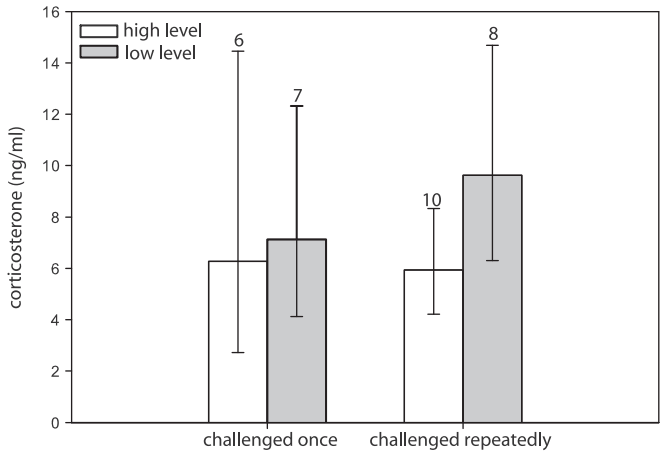

Fig. 5. Back-transformed means (±95% CI) of corticosterone levels did not differ between males that were challenged only once and males that were sampled after the final of the repeated challenges independent of intruder type. Samples sizes are given above bars.

First, in some species a single interaction between males may not be sufficient to induce a rise in testosterone. Instead, multiple such interactions may be necessary to affect hormone levels (see Oyegbile and Marler, 2005). However, in male black redstarts also multiple consecutive STIs did not lead to a change in androgens.

Second, we tested if STIs induce a losing experience which may prevent an increase or may even lead to a decline in testosterone (Goymann et al., 2007a; Kempenaers et al., 2008). The behaviors black redstarts showed towards single and repeated STIs with self-retreating low-level and high-level intruders strongly suggest that this is not a likely explanation for the absence of an androgen response during STIs in this species.

Third, the hormonal response did not differ between individuals challenged with a high- and a low-level intruder, suggesting that intrusion intensity did not influence the likelihood of inducing a winning or losing experience. In addition, the strong behavioral response to high-level intrusions does not conform to the expectation of a reduced behavioral response when losing.

Our results demonstrate that neither single nor multiple STIs result in an increase of androgens or corticosterone in black redstarts. In contrast to the absence of a hormonal response, repeated STIs induced clear behavioral responses depending on the intensity of the

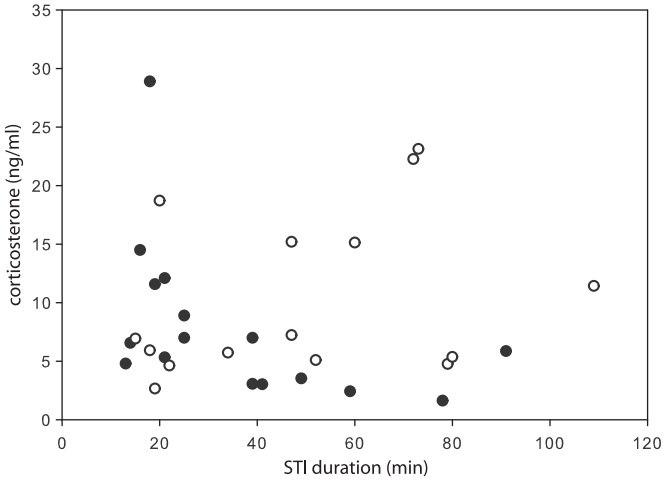

Fig. 6. Corticosterone levels of males challenged with the high-level intruder (filled symbols) were significantly negatively correlated with STI duration. Corticosterone levels of males challenged with the low-level intruder (open symbols) show no significant relationship with STI duration.

intrusion. Males challenged with different high-level intruders during three consecutive days approached the decoy faster on the third day than on the first day tested. These behavioral results are similar to those of others, who found decreased response latencies (Antunes and Oliveira, 2009) or an increase in aggressive behaviors during subsequent territorial challenges (e.g. Parmigiani and Brain, 1983; Trainor and Marler, 2001). Also, in winner-loser tests conducted with males of socially monogamous California mice (*Peromyscus californicus*) the attack latency decreased during subsequent encounters (Oyegbile and Marler, 2005). However, unlike black redstarts California mice had higher testosterone levels after the final encounter. In this respect, polygynous white-footed mice (*P. leucopus*), which also showed no change in testosterone after repeated challenges, were more similar to black redstarts (Oyegbile and Marler, 2006). Thus, in contrast to the socially monogamous black redstarts, California and white-footed mice conform to the challenge hypothesis, which predicts that socially monogamous species with paternal care should respond with an increase in testosterone during male–male challenges, whereas polygynous species without paternal care should not do so (Wingfield et al., 1990).

By using different low and high intensity intruders and by automatically removing the intruders during three consecutive days our study attempted to create a winning experience in the focal male before capturing it during the final STI on the fourth day. By using this setup we think that we can exclude that the lack of an increase in androgens during STIs in black redstarts (and most likely in other bird species that lack a testosterone response during STIs) is caused by a losing experience for several reasons.

First, the overall intensity of the behavioral response did not change over days. If focal males would have perceived the STIs as losing experience we would have expected a reduced territorial response during consecutive STIs (Hsu et al., 2006). Furthermore, the behavioral data suggest that males challenged with a high-level intruder did not perceive these challenges as a losing experience. These males approached the decoys faster during subsequent days, they spent more time very close to the decoy and they sang more songs compared to black redstarts challenged with low-level intruders. Thus, high-level intruders were probably perceived as more threatening than low-level intruders. But rather than giving up, as when losing an encounter, the males showed a stronger territorial response. Some recent studies on other songbirds point into the same direction. For example, males of the golden whistler (*Pachycephala pectoralis*) stayed closer to a decoy during a high-level intrusion than during a low-level intrusion (VanDongen and Mulder, 2008). De Kort et al. (2009) found that male banded wrens (*Thryophilus pleurostictus*) reacted more aggressively and sang more often when challenged with a median performance singer than when challenged with a high or low performance singer. Thus, the behavioral responses of birds highly depend on the intensity of a STI.

Second, also the corticosterone data of the black redstarts argue against STIs creating a losing situation. If the STIs would have caused a losing experience in black redstarts, we would have expected higher levels of corticosterone in males that were repeatedly challenged with high-level intruders. However, corticosterone levels did not differ between males that were challenged once and males that were challenged multiple times before capture. Corticosterone levels of STI challenged redstarts were similar to those of redstarts caught passively without any challenge (V. Görlich & W. Goymann, unpublished data). Furthermore, although high-level intruders evoked a stronger behavioral response and were obviously perceived as more menacing than low-level intruders, plasma corticosterone levels did not differ between intrusion intensities. In males challenged with high-level intruders, plasma corticosterone levels were even lower the longer the STI lasted. Thus, corticosterone levels either decreased during the STI or males caught later during the challenge had lower levels of corticosterone to begin with.

In summary, because black redstarts showed a stronger behavioral response towards high-level intruders than towards low-level intruders, and because there were no differences in testosterone or corticosterone levels, neither between birds challenged only once or multiple times nor between birds challenged with high-or low-level intruders, we consider it unlikely that single or multiple STIs could have created a losing experience in these birds.

Conclusions

Male black redstarts seem to be very resistant against social modulation of androgen levels during STIs. Although they are physiologically capable to mount an androgen response both during STIs and during real intrusions, they do not do so (Apfelbeck and Goymann, 2011). Here, we demonstrated that even repeated STIs did not result in an increase in androgens and that different STI intensities had a strong effect on behavior but not on hormone levels. Thus, in this species, aggressive behavior does not seem to influence plasma androgen levels and experience alone may be sufficient to induce a winning experience. Alternatively, territorial conflicts may not affect plasma androgens, but may lead to local production of neurosteroids directly in the brain (London et al., 2006) and thereby facilitate changes in behavior. Furthermore, there was no evidence that the lack of an androgen response was connected to a losing experience following STIs. Substantial behavioral evidence and hormonal data suggest that the opposite is the case, i.e. the larger the threat the more vigorously they defended their territory and they showed no increase in corticosterone levels which may have indicated a defeat.

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Chapter Four

Testosterone affects song modulation during simulated territorial encounters in male black redstarts (*Phoenicurus ochruros*).

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Abstract

It has been suggested that testosterone plays an important role in resource allocation for competitive behaviour. However, besides this general relationship, details of the interplay between testosterone, territorial aggression and signal plasticity in a natural context are largely unknown. Therefore, we studied if testosterone acts specifically on signals that communicate the motivation or ability of individuals to engage in such competitive situations, using the black redstart, a territorial songbird species as study organism. During spring, male territory holders were implanted with an androgen receptor blocker and an aromatase inhibitor to inhibit the action of testosterone or metabolites of this hormone. Controls received a placebo treatment. Three days after implantation birds were challenged with a simulated territorial intrusion. Song was recorded before, during and after the challenge. Both blocker and placebo-implanted males increased the number of elements sang in parts of their song. However, while placebo-implanted males sang one of these parts with the same maximum

frequency as in the unchallenged situation, blocker-implanted males decreased the maximum frequency of that part. Furthermore, placebo-, but not blocker-implanted males, sang shorter songs with shorter pauses between parts and the atonal part of their song with a broader frequency bandwidth in the agonistic context. To the best of our knowledge this study is the first to show that testosterone facilitates context dependent changes in song structures that may be honest signals of male quality in a songbird.

Introduction

Sexually selected signals often serve both to attract a mate and to advertise competitive abilities for example during territorial disputes (reviewed in Searcy and Nowicki 2005). Testosterone is thought to be one of the main hormones involved in adjusting such signals according to the behavioural context. For example, territorial behaviours and associated vocalisations in a wide range of male vertebrates during breeding are modulated by testosterone (reviewed in Adkins-Regan 2005). It has been suggested that testosterone might play an important role in resource allocation for competitive behaviour (reviewed in Wingfield, Moore et al. 2006; Botero, Rossman et al. 2009). From that point of view, testosterone should act specifically on signals that communicate the motivation or ability of individuals to engage in such competitive situations. However, besides this general relationship, details of the interplay between

hormones, territorial aggression and signal plasticity in a natural context are largely unknown.

With regard to this, it may be elucidating to study the song behaviour of male passerine birds. In many bird species, males modulate their song in an aggressive context (e.g. Price, Earnshaw et al. 2006; DuBois, Nowicki et al. 2009) and male as well as female listeners respond differentially to such modulations (Draganoiu, Nagle et al. 2002; Draganoiu, Pasteau et al. 2006; Illes, Hall et al. 2006; DuBois, Nowicki et al. 2011). Besides changes in the general output of song (e.g. song rate or amplitude), song modulations also occur in structural song characteristics. Those structural features describe for example song repertoire characteristics (Voigt and Leitner 2008) or song parts that are challenging to sing such as rapid broadband trills (reviewed in Podos, Lahti et al. 2009); specific song trills (Vallet, Kreutzer et al. 1997) or consistent syllables (Botero, Rossman et al. 2009). Structural song patterns have been classified as 'index signals' that honestly communicate a physical trait related to male quality (Hurd and Enquist 2005). Only very few studies revealed a capability of individuals to modulate even such physically constrained signals within narrow limits (Bee and Perrill 1996; Price, Earnshaw et al. 2006; DuBois, Nowicki et al. 2009). Thus, from a functional point of view, index signals such as structural song parameters should play an important role in the communication of competitive ability.

The steroid hormone testosterone plays an important role in the regulation of adult singing (reviewed in Harding 2004) and manipulating testosterone levels

may alter song output (measured e.g. as song rate or duration; e.g. Silverin 1980; Ketterson, Nolan et al. 1992; Hau, Wikelski et al. 2000; Boseret, Carere et al. 2006; Ritschard, Laucht et al. 2011). Whether testosterone also affects structural song parameters is less clear. In barn swallows (*Hirundo rustica*), the duration and pulse rate of the harsh 'rattle' element correlated moderately with absolute testosterone levels (Galeotti, Saino et al. 1997). Manipulation studies showed that zebra finches (*Taeniopygia guttata*) treated with testosterone decreased the fundamental frequency of harmonic stacks in their song on a long-term basis (Cynx, Bean et al. 2005). Other correlative studies or studies with testosterone treatment failed to find effects on structural song parameters (Weatherhead, Metz et al. 1993; Kunc, Foerster et al. 2006; Ritschard, Laucht et al. 2011). Studies that implant birds with testosterone may be problematic, because right after implantation testosterone is released in pharmacological doses (Fusani 2008). It is thus questionable whether manipulations within the physiological range of testosterone levels would induce similar effects. The – so far - only study in which the androgenic and oestrogenic pathways of testosterone action were blocked, failed to find effects on structural song parameters in great tits (*Parus major*, Van Duyse, Pinxten et al. 2005). Thus, to the best of our knowledge, an effect of physiological changes in testosterone on the structure of song has not been demonstrated so far.

In this study, we investigated spontaneous song and reactive singing in an aggressive context of free-living male black redstarts (*Phoenicurus ochruros*). Testosterone can modulate behaviour by either directly binding to androgen

receptors or indirectly by binding to oestrogen receptors (Balthazart 1997). We tested the role of testosterone in the modulation of song characteristics by blocking androgen receptors with flutamide and by inhibiting the conversion of testosterone to oestradiol with letrozole, an aromatase inhibitor (Cheshenko, Pakdel et al. 2008). As controls we used birds treated with placebo implants. After implantation, we first recorded the spontaneous song of territorial males in an undisturbed context and then conducted a playback experiment simulating a territorial intrusion (STI) by a foreign male. The aim of our study was twofold. First, we wanted to investigate whether black redstarts change structural song parameters in an aggressive context. Second, by blocking testosterone in a subset of birds, we attempted to determine the role of this hormone in context-dependent vocal plasticity. If testosterone is playing a key role in the resource allocation for competitive behaviour (e.g. Hau 2007), we would expect blocker-treated males to invest less in those behaviours and song patterns that are relevant in such situations. Index signals that honestly communicate a physical trait related to male quality (Hurd and Enquist 2005) are good candidates here.

Material and Methods

Black redstarts are socially monogamous song birds that establish territories after returning from their wintering grounds in March (Landmann and Kollinsky 1995). Adult (≥ 2 years) territorial male black redstarts were caught in 2009 (April 9^{th} - 27^{th}) in Upper Bavaria, Germany (N 47º, E 11º). Males were implanted with either one placebo pellet (n = 10) or two time release pellets (n = 10, Innovative

research of America, Sarasota, FL) containing the androgen receptor blocker flutamide and the aromatase inhibitor letrozole, respectively. For details on the effect of the blockers on plasma testosterone levels see Apfelbeck et al. (in prep., chapter 5). In addition, each male was colour-banded for individual recognition. All experimental procedures were approved by the governmental authorities of Upper Bavaria.

Simulated territorial intrusions were conducted three days after implantation. For the playback we used songs from 20 adult male black redstarts recorded in spring 2009 with a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660 (sampling frequency: 44.1 kHz; resolution: 16 bit). For each target male we selected a playback that was recorded at least 10 km away from the study area. Playbacks were put together using Avisoft-SASLab Pro software, version 4.51 (Raimund Specht, Berlin, Germany). Each playback consisted of 20 songs recorded from one male. Songs were filtered (1 kHz high-pass filter) and amplitude was normalized to 75 %. A playback consisted of each of two different song types (X and Y) played back in a XXYYXXYYXX sequence, with X and Y in 10 different versions. Songs were divided by pauses of 4.5 s. By repeating each sequence six times the playback had a duration of 20 minutes in total. This design resembled the natural song organisation of the species. Each playback was used only during one STI of each experimental group.

The simulated territorial intrusion (STI) experiments were performed by placing a stuffed decoy (male in full adult plumage protected by an inconspicuous

cage, three different decoys used) into the centre of the respective territories. A remote-controlled loudspeaker (Foxpro Scorpion, digital game caller, FOXPRO Inc. Lewistown, USA) was put underneath the decoy to play back the territorial song of a potential rival at a sound pressure level of 65 dB SPL at 1 m (as measured with a CEL 573.B1 Sound Level Analyzer). We only started an experiment when a male was singing. The song was recorded 10 min prior to the start of the STI, during the 20 min STI and 10 min after the STI.

The song was analysed using Avisoft-SASLab Pro software, version 4.51. Recordings were visualized in spectrograms (settings: sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50 %). We determined the number of songs by visual inspection and selected songs of sufficient quality (low background noise) for further sound analysis. Each song of black redstarts can be divided into three distinct parts (part A, B and C, see Fig. 1 and e.g. Cucco and Malacarne 1999) with a pause of varying length between part A and B. We measured the duration of part A, B, C, the total song and the duration of pauses between A and B (Fig. 1). We counted the number of elements of part A and C (mean of max. 20 songs). We also determined the frequency bandwidth and the maximum frequency of part A, B and C using the automatic parameter measurement function (threshold -20 dB) in Avisoft (mean of max. 10 renditions of high-quality songs).

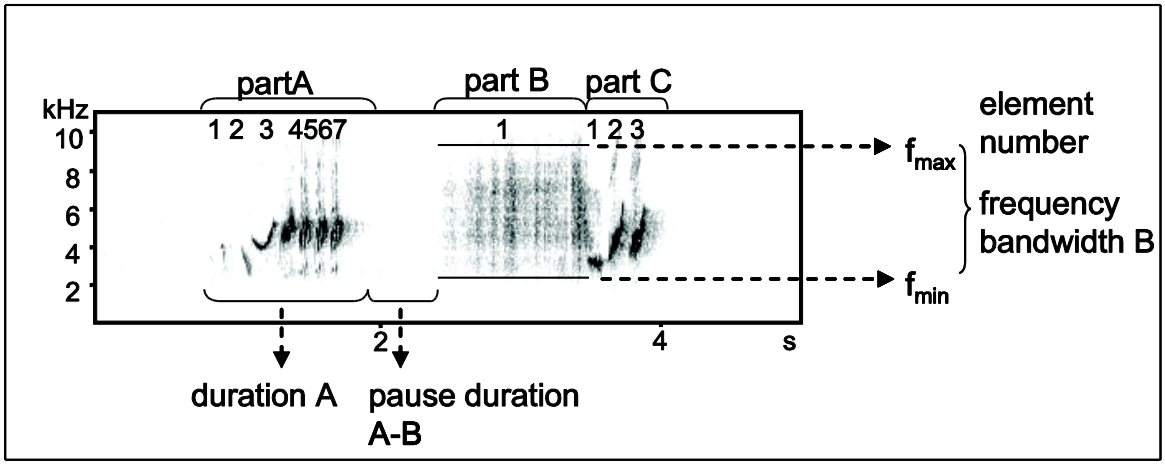

Figure 1: A song of a black redstart illustrating the acoustic measures analyzed (Spectrogram: Avisoft-SASLab Pro, sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50 %).

Data were analysed with R version 2.9.1 (Development Core Team 2009). Song before, during, and after the STI was analysed using general linear mixed models with bird identity as a random effect to control for repeated measures. We analysed whether the dependent variables (number of songs, song duration, duration of part A, B, C and the pause between part A and B, the number of elements in part A and C and maximum frequency and bandwidth of all parts) were influenced by the blocker treatment, part of the STI and their interaction. In all cases, dependent variables where transformed if assumptions of normality and/or equality of variances were not met. Significance was accepted at $\alpha \leq 0.05$.

Results

Males sang significantly fewer songs during the STI than when singing spontaneously before and after the STI (Table 1, Fig. 2).

Song duration significantly changed in control males, with songs during the STI being shorter than before or after the STI. In blocker-implanted males, song duration remained constant before, during and after the STI (Table 1). The shortening of the song in control males was mainly due to a significantly shorter pause between song part A and B (Table 1), because the durations of the three song parts (A, B and C) did not differ significantly before, during and after the STI (Table 1). Thus, even though placebo-implanted males sang shorter overall-songs during the STI than blocker-implanted males, this reflected a higher signal density because of shorter pauses.

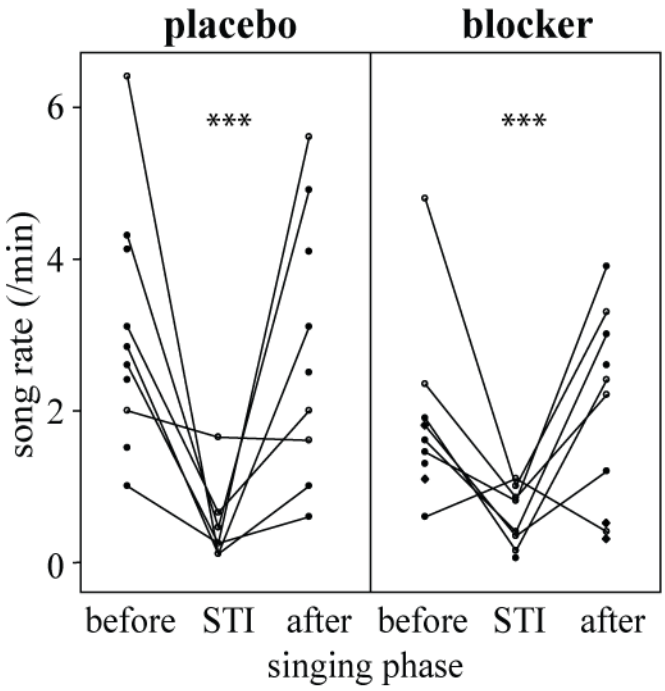

Figure 2: Song rate (mean ± 95 % confidence intervals) before, during and after the STI, separated for males treated with flutamide and letrozole (‘blocker’, n = 10) and placebo treated males (‘control’, n = 10). For details on song measurements and statistics see text.

Table 1. Linear mixed model results. Part is a within-subjects factor with three levels: before STI (= spontaneously sung songs), during STI (playback and decoy present) and after STI (directly after removal of playback and decoy). Treatment is a between-subjects factor with two levels: placebo implanted vs. blocker implanted males. To control for repeated measures the ID of each territory owner was included as random intercept. Significant results are highlighted in bold.

	treatment	**part**	**part*treatment**
song rate	$F_{1,18} = 2.3$	**$F_{2,30} = 23.6$**	$F_{2,30} = 1.3$
	p = 0.1	**p < 0.0001**	p = 0.3
song duration	$F_{1,18} = 0.2$	**$F_{2,30} = 6.7$**	**$F_{2,30} = 3.8$**
	p = 0.6	**p = 0.004**	**p = 0.03**
duration A	$F_{1,18} = 0.7$	$F_{2,30} = 2.3$	$F_{2,30} = 1.4$
	p = 0.4	p = 0.1	p = 0.3
duration B	$F_{1,18} = 0.2$	$F_{2,30} = 2.0$	$F_{2,30} = 0.4$
	p = 0.6	p = 0.2	p = 0.6
duration C	$F_{1,18} = 0.4$	$F_{2,30} = 0.9$	$F_{2,30} = 0.2$
	p = 0.6	p = 0.4	p = 0.8
duration pause A-B	$F_{1,18} = 1.2$	**$F_{2,30} = 7.6$**	$F_{2,30} = 1.0$
	p = 0.3	**p = 0.002**	p = 0.4
no of elements in A	$F_{1,18} = 0.6$	**$F_{2,30} = 23.1$**	$F_{2,30} = 1.1$
	p = 0.6	**p < 0.0001**	p = 0.4
no of elements in C	$F_{1,18} = 0.04$	**$F_{2,30} = 12.2$**	$F_{2,30} = 0.9$
	p = 0.8	**p < 0.0001**	p = 0.4
freq bandwidth A	$F_{1,18} = 1.5$	$F_{2,30} = 1.1$	$F_{2,30} = 2.1$
	p = 0.2	p = 0.4	p = 0.1
max frequency A	$F_{1,18} = 1.4$	**$F_{2,30} = 3.9$**	**$F_{2,30} = 5.1$**
	p = 0.3	**p = 0.03**	**p = 0.01**
freq bandwidth B	$F_{1,18} = 3.7$	**$F_{2,30} = 4.4$**	**$F_{2,30} = 5.4$**
	p = 0.07	**p = 0.02**	**p = 0.009**
Max frequency B	**$F_{1,18} = 6.6$**	**$F_{2,30} = 3.6$**	$F_{2,30} = 2.0$
	p = 0.02	**p = 0.04**	p = 0.2
freq bandwidth C	$F_{1,18} = 0.1$	$F_{2,30} = 0.9$	$F_{2,30} = 0.09$
	p = 0.7	p = 0.4	p = 0.9
max frequency C	$F_{1,18} = 0.2$	$F_{2,30} = 2.1$	$F_{2,30} = 0.2$
	p = 0.7	p = 0.1	p = 0.8

Both placebo- and blocker-implanted males sang significantly more elements in song part A and C during and after the STI than before the STI (Table 1, Figs. 3a, b). This element increase resulted from an increase in the number of elements of the trilled phrases of part A or C, respectively (Fig. 1). By definition, part B did not change with respect to this measure because it consisted of one element only (Fig. 1).

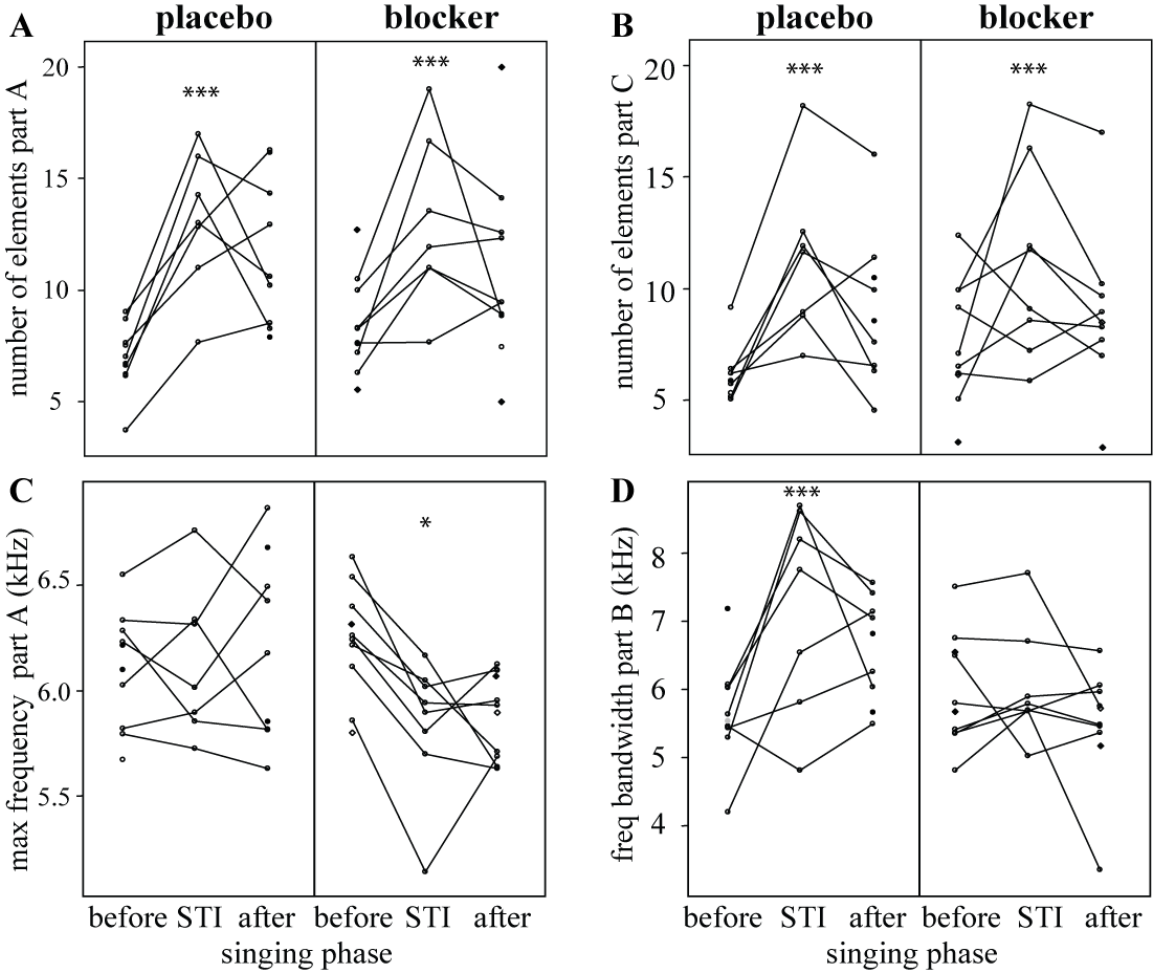

Figure 3: Structural song measures (mean ± 95 % confidence intervals) before, during and after the STI, separated for males treated with flutamide and letrozole (‘blocker’, n = 10) and placebo treated males (‘control’, n = 10). For details on song measurements and statistics see text.

Blocker-implanted males sang part A with a significantly lower maximum frequency during and after the STI than before the STI. In contrast, the maximum frequency of part A did not change before, during and after the STI in placebo-implanted males (Table 1, Fig. 3c). As a consequence of theses changes in maximum frequencies of part A, blocker implanted males tended to sing this part with a lower frequency bandwidth after the STI than placebo-implanted males. Both treatment groups sang part B with a significantly higher maximum frequency during the STI than before the STI. Furthermore, the maximum frequency of this part tended to remain high after the STI in placebo-implanted males but not in blocker-implanted males (Table 1). Consequently, placebo-implanted males sang part B with a significantly larger frequency bandwidth during and after the STI than before the STI, while frequency bandwidth of part B did not change in blocker implanted males (Table 1, Fig. 3d). Maximum frequency and the frequency bandwidth of part C did not change in response to the STI or blocker treatment (Table 1).

Discussion

In this study, we explored the role of testosterone and its oestrogenic metabolites in modulating song characteristics in black redstarts in a spontaneous and a reactive context. Territorial males of both blocker and control groups responded to the STI with changes in their song: besides decreasing the song rate, they also increased the number of elements in part A and C while keeping the length of these parts constant. However, while placebo-implanted males maintained the maximum frequency of song part A despite the higher element rate at the same

level than before the STI, blocker implanted males sang part A with a lower maximum frequency. In addition, placebo-implanted males increased the frequency bandwidth of part B during the aggressive context, while blocker-implanted males did not do so. Placebo-implanted males also sang shorter overall songs due to a shorter pause between part A and B.

In summary, territorial males of both groups did change structural song parameters in an aggressive context. The increase in the number of elements sung in part A and C may indicate that black redstarts increased the performance of these parts during the aggressive context. However, in blocker-implanted males this increase in the number of elements in part A was associated with a decrease in its maximum frequency (and they therefore tended to sing this song part with a lower frequency bandwidth than during spontaneous song). This suggests that they could not increase the number of elements and maintain the frequency at the same time or that they invested less into the production of these signals than did control birds. In addition, in contrast to placebo-implanted males they did not increase the frequency bandwidth of song part B. Part B consists only of a single noisy song element (Fig. 1). There are good reasons to assume that such atonal song elements are not produced by the syrinx but by modulating the airflow in the vocal tract (reviewed in Riede and Goller 2010). Accordingly, placebo-implanted birds may sing with a higher air pressure and thus louder than blocker-implanted males. As a consequence, a broader range of frequencies is “broadcasted” in control males than in blocker-implanted males. Alternatively, a broader frequency range might be achieved by an increase in beak opening (e.g.

Hoese, Podos et al. 2000). In barn swallows, the song characteristics of a similar harsh or noisy element, the rattle, were correlated with testosterone concentration (Galeotti, Saino et al. 1997). Changing the acoustic properties of such elements within limits may be interpreted as a way to increase their signal value as described in the framework of index signals.

With regard to trilled parts, it has been suggested previously that the production of repeated (trilled) syllables with a high frequency bandwidth is challenging (reviewed in Podos, Lahti et al. 2009) and probably an honest signal of male quality (Illes, Hall et al. 2006; Ballentine 2009). Females of some species prefer songs sung with a high trill rate and broad frequency bandwidth (Draganoiu, Nagle et al. 2002; Ballentine, Hyman et al. 2004). Furthermore, swamp sparrows (*Melospizia georgiana*) increase both trill rate and frequency bandwidth in response to simulated territorial intruders (DuBois, Nowicki et al. 2009). The song changes of black redstarts during aggressive encounters fit well within this picture: redstarts changed structural song parameters that have been suggested to be physically challenging in other species.

Our experiment suggests that some, though not all song response measures in an aggressive context are mediated by testosterone directly or via its conversion to oestradiol. Blocking these hormones particularly affected structural song measures. This may indicate that testosterone represents an underlying mechanism allowing the modification of ‘index signals’ such as trill rate or frequency measures. Similar results were recently reported for singing mice (Pasch, George et al. 2011) and may thus reflect a general mechanism in

vertebrates. In birds, such a modification within limits may be achieved for example by modifying properties of the syrinx, a testosterone and oestrogen sensitive organ (Wade and Buhlman 2000; Veney and Wade 2004; Veney and Wade 2005), or the beak muscles (Hoese, Podos et al. 2000). In addition, the neuronal coordination of singing might be affected by changes in testosterone levels, too (reviewed in Balthazart, Charlier et al. 2010).

In conclusion, our study demonstrates that manipulating the effects of testosterone within a physiological range had effects on both song output and structural song measures of black redstarts during competitive situations. We conclude that testosterone may affect both the signal parameters indicating the motivation and the ability to engage in competitive interactions such as territorial disputes. This might be achieved by effects of testosterone on the neuronal and physiological capabilities to produce certain song elements depending on the behavioral context. This very nicely illustrates that hormones may not generally change the likelihood of a behavior, but only does so in a context-dependent manner (Monaghan and Glickman 1992).

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Chapter Five

Testosterone does not drive territorial aggression, but emphasizes vocal behaviours in the territorial response in breeding black redstarts, *Phoenicurus ochruros*

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Abstract

Many studies in behavioural endocrinology attempt to link territorial aggression with testosterone, but the exact relationship between testosterone and territorial behaviour is still unclear and results for different species seem conflicting. The degree to which testosterone facilitates territorial behaviour is particularly little understood in species that defend territories during breeding and outside the breeding season, when plasma levels of testosterone are low. Here we suggest that species that defend territories for extended periods of time and also independent of reproduction may have lost the direct regulation of territorial behaviour by androgens to avoid the costs of testosterone. Only sexually relevant components of this behaviour may be under control of sex steroids during breeding. We treated territorial male black redstarts (*Phoenicurus ochruros*) with an antiandrogen and an aromatase inhibitor during the breeding season to block both the direct and indirect effects of testosterone. Three and ten days after the treatment, implanted males were challenged with a simulated

territorial intrusion. The territorial response as a whole was not reduced, but the treatment changed the emphasis of territoriality: blocker-implanted males invested more in behaviours addressed directly towards the intruder, whereas placebo-treated males put most effort into their vocal response, a component of territoriality that may be primarily directed towards their mating partner rather than the opponent. In combination with previous findings, these data suggest that overall territoriality may be decoupled from testosterone in male black redstarts. However, high levels of testosterone during breeding may facilitate context dependent changes in song structure.

Introduction

In a reproductive context, testosterone and its metabolite oestradiol are considered major hormones facilitating territorial behaviour and the associated vocalizations in a wide range of male vertebrates (e.g. Lincoln, Guinness et al. 1972; Wingfield, Hegner et al. 1990). Particularly in birds, seasonal peaks in testosterone closely match with periods of intense male-male competition for territories and mates (Wingfield, Hegner et al. 1990). Furthermore in songbirds, testosterone and oestradiol play an important role in the activation of song during the breeding season (reviewed in Ball and Balthazart 2010). Brain areas involved in the production and learning of song and neural areas associated with sexual arousal and aggression express receptors for androgens and oestrogens as well as the enzyme aromatase, which converts testosterone into oestradiol (Gahr and Metzdorf 1997; Metzdorf, Gahr et al. 1999). Hence, testosterone can modulate

behaviour either directly or indirectly by conversion to oestradiol (Schlinger and Callard 1990; Balthazart 1997).

Sex steroids such as testosterone and oestradiol orchestrate physiological, morphological and behavioural changes important for reproduction (e.g. Lincoln, Guinness et al. 1972). Thus, a close link between the expression of territorial behaviour and testosterone ensures that this behaviour is only expressed in the appropriate context (Adkins-Regan 2005), as maintaining high levels of aggression is energetically costly and may impair survival (Dufty 1989; Wingfield, Lynn et al. 2001). However, especially in songbird species it is quite common that males also defend territories outside the breeding season, when testes are regressed and testosterone levels are low (Schwabl and Kriner 1991; Wingfield 1994; Canoine and Gwinner 2002; Hau and Beebe 2011). When a behaviour is expressed over a long time period or even throughout the year its control may be decoupled from hormones (e.g. Adkins-Regan 2005). Hence, in cases when territorial behaviour occurs during most of the year it may be independent of testosterone. Although only few species have been studied in this regard so far, these studies suggest that - at least during the breeding season - testosterone plays a role in the regulation of territorial behaviour also in species that defend territories in reproductive and non-reproductive contexts (Schwabl and Kriner 1991; Wingfield 1994; Canoine and Gwinner 2002; Hau and Beebe 2011; Marasco, Fusani et al. 2011). However, the degree to which testosterone facilitates territoriality appears to differ between species. Three potential mechanisms seem likely.

First, in some species the degree of territorial aggression differs between breeding and non-breeding context with males expressing only low levels of territorial aggression outside the breeding season. This low-intensity territorial behaviour may be independent of testosterone and testosterone intensifies territoriality during reproduction. This has been shown to be the case, for example, in male mountain spiny lizards (*(Sceloporus jarrovi,* Moore 1986; Moore 1988) and European nuthatches (*Sitta europea*, Landys, Goymann et al. 2010).

Second, testosterone may facilitate territoriality in breeding and non-breeding contexts, but may be derived from different sources during breeding and non-breeding. Song sparrows, *Melospiza melodia*, show similar levels of territorial aggression during the breeding and the non-breeding season (Wingfield 1994). Here, territorial behaviour was facilitated by conversion of testosterone to oestradiol (Soma, Tramontin et al. 2000). In the non-breeding season, when circulating levels of testosterone are low, testosterone may be produced directly in the brain by conversion of dehydroepiandrosterone (DHEA) to testosterone or oestradiol. DHEA is of nongonadal origin and its plasma levels are elevated during the non-breeding season in song sparrows (Soma and Wingfield 2001). It is surprising though, that several components of territorial behaviour in song sparrows seem to be driven by oestradiol during the non-breeding season, but not during breeding: the treatment with fadrazole, an aromatase inhibitor, clearly affected several measures of territorial behaviour during non-breeding, but had little effect during breeding (Soma, Sullivan et al. 2000).

Third, sex steroids may activate exclusively those components of territorial behaviour that are relevant in a breeding context only. This may be the case in most songbird species studied so far (Table 1) because a strong overall effect of testosterone on territorial behaviour is the exception rather than the rule. For example, none of the studies applying pharmacological blockers inhibiting the (direct and/or indirect) effects of testosterone reported that blocker-implanted birds lost their territory (Table 1). Furthermore, in most cases the treatment only reduced some aspects of the territorial behaviour (Schwabl and Kriner 1991; Hau, Wikelski et al. 2000; Canoine and Gwinner 2002; Sperry, Wacker et al. 2010). In one case there was even no effect at all on any of the behaviours measured (Moore, Walker et al. 2004). In addition, in studies where the blockers had an effect on territorial behaviour, the effect was usually found during the breeding season, but not during non-breeding (Schwabl and Kriner 1991; Canoine and Gwinner 2002; Hau and Beebe 2011). Territoriality consists of a variety of behaviours including vocalizations (song, calls), spatial behaviours, threat displays and direct aggression. Similar to courtship displays (Fusani, Gahr et al. 2001), these different components may be facilitated by distinct (hormonal) pathways (Schwabl and Kriner 1991; Van Duyse, Pinxten et al. 2002; Sperry, Wacker et al. 2010). During the breeding season, testosterone may specifically activate those aspects of the territorial response that are relevant in a reproductive context, i.e. involve signalling to females. For example, in the grey partridge *(Perdix perdix)* testosterone manipulations affect the quality of the rusty

gate call and its salience for females (Beani and Dessi-Fulgheri 1995; Beani, Panzica et al. 1995).

The aim of this study was to investigate the role of testosterone and its metabolite oestradiol in the regulation of territorial behaviour in male black redstarts (*Phoenicurus ochruros*). Males of this species are highly territorial in spring when their testosterone levels are elevated, but also in autumn when testosterone is basal (Apfelbeck and Goymann 2011, chapter 2). Similar to other songbirds males express androgen receptors in nuclei of the song control system, and androgen and estrogen receptors as well as aromatase in pre-optic and hypothalamic areas (based on in situ hybridization studies, Apfelbeck et al., in prep., chapter 1). However, unlike some other species (reviewed in Goymann 2009) male black redstarts do not increase testosterone during agonistic encounters with other males or during simulated territorial intrusions (STI) with a male decoy (Apfelbeck and Goymann 2011; Apfelbeck, Stegherr et al. 2011, chapters 2 and 3).

In male black redstarts non-vocal territorial behaviours do not differ between the breeding and the non-breeding season and thus are not correlated with circulating testosterone levels (Apfelbeck and Goymann 2011, chapter 2). However, context - dependent structural changes in the song seem to depend on testosterone or oestradiol during breeding (Apfelbeck et al., submitted, chapter 4). We, thus, hypothesized that territorial behaviour as such should be decoupled from the control of sex steroids in this species. Instead, only those components of territoriality (e.g. song structure) that are particularly relevant in a breeding

context should be influenced by sex steroids. We implanted male black redstarts with the anti-androgen flutamide and the aromatase inhibitor letrozole and challenged them with a simulated territorial intrusion (using a mounted decoy and audio-playback of black redstart song), three and ten days after implantation. We predicted that the ability of blocker-treated males to defend a territory should not differ from that of control males during the breeding season. However, based on our previous findings (Apfelbeck et al., submitted, chapter 4), males implanted with blockers should invest less into vocal performance than placebo-implanted males.

Table 1. Effects of blocker treatment in different species.

species	inhibited	days	song output	song structure	calls	approach latency	closest approach	time spent close	latency attack	flights, chases	threat displays	loss of territory	references
breeding season													
European stonechat	AR/ Aro	7-17					**yes**		**yes**			no	Canoine&Gwinner, 2001 Marasco et al., 2011
European robin	AR	8-14 and	no			no	no	no				no	Schwabl & Kriner,
		18-25	no			**yes**	no	no				no	1991
Song sparrow	AR pre-breeding	18	no			no	no	no		**yes**		no	Sperry et al., 2010
Song sparrow	AR breeding	18	no			no	no	no		no		no	Sperry et al., 2010
Song sparrow	Aro	24hrs and	no			no	no	no		no		no	Soma et al., 2000
		8-10	no			no	no	no		no		no	
Red-winged blackbird* (polygynous)	AR/Aro	2-5	**no (1)**		no					no		**no (2)**	Beletsky et al., 1990[1]
Red-winged blackbird*	AR	4-12	no		no					no		no	Beletsky et al., 1990[1]
Great tit	AR/Aro	2-5	**yes (3)**	no (4)								no	VanDuyse et al., 2005[1]
Spotted antbird	AR/Aro	8	**yes (5)**		**Less snarls**						no	Lab study	Hau et al., 2000
Rufous-collared sparrow	AR/Aro	7-13	no			no	no	no		no		no	Moore et al., 2004
non-breeding season	**inhibited**	**days**	**song output**	**song structure**	**calls**	**approach latency**	**closest approach**	**time spent close**	**latency attack**	**flights, chases**	**threat displays**	**loss of territory**	**references**
European stonechat	AR/Aro	7-17					no		no			no	Canoine&Gwinner, 2001
European robin	AR	6 and	no			no	no	no				no	Schwabl & Kriner,
		31-39	no			no	no	no				no	1991
Song sparrow	Aro	24hrs and	no,			no,	**yes**	no,		**yes**		no	Soma et al., 2000a
		9-12	**yes**			**yes**	**yes**	**yes**		**yes**		no	Soma et al., 2000b
Song sparrow	AR/Aro	7	no,			no,	no,	no,		no,		no	Soma et al., 1999
		and 30	**yes**			**yes**	**yes**	**yes**		**yes**		no	
Spotted antbird	AR/Aro	8	no		no						no	lab study	Hau and Beebe, 2011

The column “inhibited” indicates the type of blocker treatment. AR: androgen receptors were blocked; Aro: the enzyme aromatase and thus the conversion of androgens to oestrogens was blocked. All except the last two species were temperate-zone breeding birds. Spotted-antbirds and rufous-collared sparrows breed in the tropics. Most of the studies assessed territorial behaviour by challenging implanted territory owners with simulated territorial intrusions, except two studies which quantified spontaneous aggression (*).

1. More vocalizations in general.
2. Some males lost parts of their territories.
3. The likelihood of dawn song was reduced.
4. There was no effect on song duration or repertoire size.
5. They sang less spontaneous song, less song towards females and during STIs.

Material and Methods

Adult (≥ 2 years) male black redstarts were caught in 2009 between April 9th and 27th in Upper Bavaria (N 47º, E 11º, 500-600m above sea level) with mealworm-baited ground traps. Birds were lured to the traps by broadcasting playbacks with the species' song of short duration (< 2min). We remotely muted the loudspeaker as soon as the territory owners approached the traps. Conspecific playback does not influence testosterone levels in territorial male black redstarts (Apfelbeck & Goymann, 2011, chapter 2). Upon capture we took several biometric measurements and implanted males alternately with either one placebo pellet (n = 10) or two time release pellets (n = 10) containing the androgen receptor blocker flutamide and the aromatase inhibitor letrozole, respectively (21 day release: 1.5 mg per pellet; release rate 71 µg/day; Ø = 3.2 mm, Innovative Research of America, Sarasota, FL). Letrozole inhibits cytochrome p450 aromatase (CYP 19). This enzyme is important for the conversion of testosterone to estrogen (Cheshenko, Pakdel et al. 2008). Thus, by combining flutamide and letrozole, it is possible to block direct and indirect effects of androgens on behaviour. Implants were inserted subcutaneously with a pair of tweezers through a small incision in the skin on the back between the wings. The incision was sealed with tissue glue (Nexaband; World Precision Instruments).

Control and experimental groups did not differ significantly in body mass ($t = 1.52$, $df = 17.9$, $p = 0.15$), length of the right tarsus ($t = -0.25$, $df = 12.5$, $p = 0.8$), length of the right wing ($t = 0.25$, $df = 14.2$, $p = 0.8$) and cloacal protuberance (CP) volume ($t = -0.17$, $df = 13.1$, $p = 0.9$), which was estimated by

calculating the volume of a cylinder ($V=\pi*(CP\ width/2)^2*CP\ height$). Each male was banded with a numbered aluminum ring (Vogelwarte Radolfzell) and a unique combination of three colour rings for individual recognition. Measuring, ringing and implanting the birds took no longer than 25 min after which the males were released onto their territories.

To assess if the blocker treatment was effective we caught another set of males with the same method as described above in April 2009 and 2010, took a blood sample immediately upon capture (within 5 min) and then brought them to the laboratory. In 2009 five males were caught, implanted with flutamide and letrozole and bled a second time three days after implantation. In 2010 we caught another eight males that were bled upon capture, either implanted with flutamide and letrozole or flutamide alone and bled again three and ten days after implantation. In all cases pellets were still visible when we took the blood samples. Males were held in individual cages under simulated natural photoperiod and released onto their respective territories after the last blood sample.

Playback stimuli

Songs used as playbacks were recorded in spring 2009 with a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660 (sampling frequency: 44.1kHz; resolution: 16 bit) from 20 different males that were at least 10 km away from our focal males. Playbacks were created using Avisoft Saslab pro software version 4.51 (Raimund Specht, Berlin, Germany). Each playback consisted of 20 songs recorded from one male. Songs

were filtered (1 kHz high-pass filter) and normalized to the same amplitude (75%). A playback consisted of each of two different song types played back in a XXYYXXYYXX sequence, with X and Y in 10 different versions. Songs were divided by pauses of 4.5 s. By repeating each sequence six times the playback had a duration of 20 minutes in total. This design resembled the natural song organization of the species. Each playback was used only once in each experimental group, but the same playbacks were used during the STIs on day 3 and 10. Thus, each male was challenged with two different playbacks.

Simulated territorial intrusion

To assess the effect of the blocker treatment on territorial behavior we performed STI experiments three and ten days after implantation by placing a stuffed decoy into the centre of the territory of a focal male and playing back black redstart song as described above. As decoys we used three different stuffed males in full adult plumage that were protected by an inconspicuous cage made of a wire frame and mist net material and mounted on a tripod. A string attached to the wire frame allowed to remotely remove the decoy by pulling the string from a distance of about 30 m into a plastic cylinder below the wire frame. We put a remote-controlled loudspeaker (Foxpro Scorpion, digital game caller, FOXPRO Inc. Lewistown, USA) underneath the decoy to play back the territorial song of a potential rival at a sound pressure level of 65 dB SPL at 1 m (as measured with a CEL 573.B1 Sound Level Analyser). The behavioural response of male black redstarts to simulated territorial intrusions varies from moving to an exposed singing post and increasing the song output to approaching the decoy and threat

posturing, which – in some cases – may cumulate into an attack (Landmann and Kollinsky 1995; Apfelbeck and Goymann 2011). Therefore, we recorded the following behaviours of the territory owner during the STI for 20 min: (1) latency to respond to the STI either by singing or approaching the decoy, (2) the first time the male entered the area of 5 m around the decoy, (3) the time the male spent within 5 m of the decoy, (4) the time the male spent within 10 m of the decoy (5) the time the territory owner spent with its feathers fluffed and (6) the number of head nods. The latter two behaviours are typical threat postures of male black redstarts (Landmann and Kollinsky 1995). Furthermore, we noted whenever the male attacked the decoy. During the whole time we also recorded the song of the territory owner using a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660. Usually we could determine the location of the male during the whole STI, however, sometimes it was hidden from view and we could not correctly record head nodding and fluffing behaviour. Therefore, we also noted when we knew the location of the bird but could not see it. After 20 min the playback was remotely muted and the decoy removed and the behaviour of the territory owner observed for another 10 min.

Song analysis

Song was analyzed using Avisoft-SASLab Pro software, version 4.51. Recordings were visualized in spectrograms (settings: sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50 %). We determined the number of songs by visual inspection and selected songs of sufficient quality (low background noise) for further sound analysis. Each song of black redstarts can

be divided into three distinct parts (part A, B and C, see Fig. 1 and e.g. Cucco and Malacarne 1999) with a pause of varying duration between part A and B. We measured the duration of part A, B, C, the total song and the duration of pauses between A and B (Fig.1). We counted the number of elements of part A and C (mean of max. 20 songs). We also determined the frequency bandwidth and the maximum frequency of part A, B and C using the automatic parameter measurement function (threshold -20 dB) in Avisoft (mean of max. 10 renditions of high-quality songs).

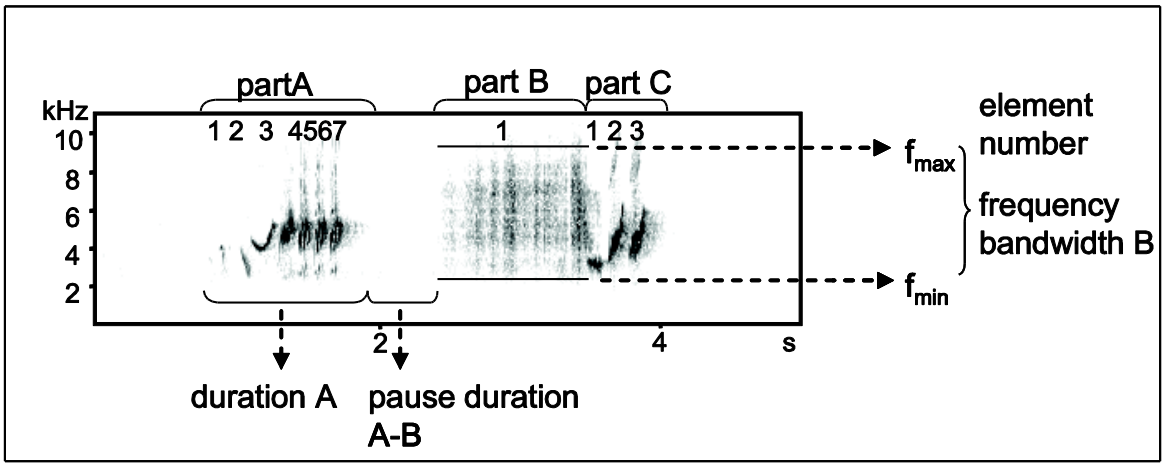

Figure 1: A song of a black redstart illustrating the acoustic measures analysed (Spectrogram: Avisoft-SASLab Pro, sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50 %).

Plasma separation and hormone analysis

Blood samples were kept cool until centrifugation at 10000 g/min speed for 10 min. Plasma was stored in 500 µl ethanol at -80 °C. Testosterone concentration was determined by direct radioimmunoassay following the procedure described

in Apfelbeck and Goymann 2011 (chapter 2) and Goymann, Geue et al. 2006. Mean ± SD efficiency of the extraction with dichloromethane was 93 ± 3 % for the samples collected in 2009 and 85 ± 5 % for those collected in 2010. Samples were measured in duplicates and samples from 2009 and 2010 were measured in separate assays. The lower limit of detection of the assay was determined as the first value outside the 95 % confidence intervals for the zero standard (B_{max}) and was 2.6 (2009) and 4.5 (2010) pg/ml. The intra-assay coefficients of variation were 1.2 % (2009) and 2.9 % (2010), respectively. The inter-assay variation was 3.2 %. As the testosterone antibody showed significant cross-reactions with 5a-dihydrotestosterone (44 %) our measurements may include a fraction of this additional androgen.

Statistical analysis

Data analysis was done with R version 2.9.1 (Development Core Team 2009). Behavioural data and measures of song structure were analysed with linear mixed models for the effects of treatment and day after implantation. We analysed the behaviour during and after the STI separately. To control for repeated measures we included bird ID as random effect. After the STI we compared the time spent within 10 m of the decoy instead of 5 m as most males left the immediate surroundings of the decoy and went to higher singing posts. Also, as most males stopped feather fluffing after the decoy was removed, we only analysed the number of head nods after the STI. When analysing the time within 5 m during the STI we included response latency as covariate in the models. When analysing treatment effects on the number of head nods and time spent feather fluffing we controlled for differences in the total amount of time we

actually saw the bird. When analysing treatment effects on song structural parameters we included the average duration of a song part as covariate in our models.

In all cases we started with full models and removed interactions if they were above $\alpha > 0.1$. Experimental factors were always retained in the models. To control for repeated measures we included bird id as random effect. Dependent variables were transformed if assumptions of normality and/or equality of variances were not met. Significance was accepted at $\alpha \leq 0.05$. Sample sizes may deviate from 20 males in total as not all males sang during the STI and depending on the quality of the recording.

Results

As expected, the combined implantation of flutamide and letrozole significantly increased plasma testosterone levels within three days as compared to levels before implantation (paired t-test, $n = 9$, $t = -3.4$, $p = 0.01$, Table 2) and testosterone levels were still significantly elevated after ten days (Wilcoxon test, $U = 0$, $p = 0.003$, Table 2), suggesting that the blocker treatment effectively inhibited the aromatization of testosterone to estrogen which in birds negatively feeds back on the production of testosterone (Soma, Sullivan et al. 1999). Testosterone levels were always well within the range of levels observed in unmanipulated birds (Apfelbeck and Goymann 2011), chapter 1). Similar to other studies flutamide implants alone did not increase or decrease testosterone levels within three and ten days (Sperry, Wacker et al. 2010; Table 2).

Table 2. Plasma testosterone levels (ng/ml ± 95 % CI) before implantation, and three and ten days after implantation of either flutamide alone or flutamide and letrozole combined.

testosterone (ng/ml)	**before**	**3 days after**	**10 days after**
flutamide / letrozole	1.02 ± 0.4 n = 9	5.02 ± 2.0 n = 9	5.9 ± 2.1 n = 4
flutamide	2.2 ± 6.6 n = 4	2.2 ± 3.4 n = 4	1.2 ± 1.5 n = 4

Territory maintenance

All control- and blocker-implanted males retained their territories during the period when the blocker treatment was effective (~ 3 weeks). In fact, most of the males, regardless of treatment, still defended the same territory during autumn, i.e. 6 months later, before they migrated to their wintering grounds (placebo: 9 out of 10, blocker: 8 out of 10).

Behaviour during STI

There was no overall effect of the blocker treatment on non-vocal territorial behaviours. However, some of the non-vocal behaviours shown in response to the STIs were reduced in blocker-implanted males during the STI at day 10 compared to the STI at day 3. In contrast, placebo-implanted males did not show such a change in non-vocal territorial behaviours with day after implantation. Blocker-implanted males spent more time within 5 m of the decoy during the STI at day 3 than during the STI at day 10. In placebo-implanted males time spent within 5 m of the decoy did not change from day 3 to day 10 (treatment: $F_{1,18}$ =

0.04, p = 0.8; day: $F_{1,15} = 1.6$, p = 0.2; interaction: $F_{1,15} = 5.1$, p = 0.04; Fig. 2). Also, blocker-implanted males spent more time with their feathers fluffed during the STI on day 3 than on day 10. No such change in feather fluffing occurred in placebo-implanted males (treatment: $F_{1,18}$=1.8, p=0.2, day: $F_{1,15}$=12.9, p = 0.003, interaction: $F_{1,15}$=7.5, p = 0.02, Fig. 2).

On the other hand, the latency to approach the decoy, the number of head nods and the flights over the decoy did not differ between placebo- and blocker-implanted males and did not change with day after implantation (approach latency: treatment: $F_{1,18} = 0.15$, p = 0.7, day: $F_{1,16} = 0.14$, p = 0.7; interaction: $F_{1,16}$ = 0.37, p = 0.6; head nods: treatment: $F_{1,18} = 0.1$, p = 0.7, day: $F_{1,15} = 3.0$, p = 0.1, interaction: $F_{1,15} = 2.0$, p = 0.2; flights: treatment: $F_{1,18} = 1.6$, p = 0.2; day: $F_{1,16}$ = 2.3, p = 0.1; interaction: $F_{1,16} = 1.9$, p = 0.2).

Three males attacked the decoy during the STI on day 3. All of them were blocker-implanted males. On day 10 only one of these males attacked the decoy. Thus, during the STI on day 3 blocker-implanted males showed a stronger non-vocal response than on day 10, because they spent more time close to the decoy and threat signalling (Table 4) and some of them also attacked the decoy. In placebo-implanted males the non-vocal response did not significantly differ between the STIs on day 3 and 10.

Males of both treatment groups sang significantly more songs during the STI on day 10 than on day 3 (treatment: $F_{1,18} = 0.04$, p = 0.8; day: $F_{1,16} = 13.4$, p = 0.002 interaction: $F_{1,16} = 0.4$, p = 0.5; Fig. 2). Placebo-implanted males sang significantly shorter songs on day 3 than on day 10 (Table 3, Fig. 3). This was

mainly due to a shorter pause duration between part A and B on day 3 than on day 10 (Table 3, Fig. 1, and Fig. 3). There was no clear change in blocker implanted males for both measures (Table 3, Fig.3). Placebo-implanted males sang part B with a broader frequency bandwidth and with a longer duration on day 3 than on day 10, while there was no clear change in blocker-implanted males (Table 3, Fig. 4). Furthermore, while the relationship between frequency bandwidth and duration of part B was positive in placebo-implanted males, it was negative in blocker-implanted males (Fig. 4). When controlling for the duration of part A, males of both treatment groups tended to sing it with fewer elements on day 10 than on day 3 (Table 3). Thus, both treatment groups sang more songs, but with a lower performance (longer songs with longer pause durations between part A and B, less elements in part A), during the STI at day 10 than at day 3. However, the decrease in song performance was much more pronounced in placebo- than in blocker-implanted males.

Table 3. Linear mixed model results for the effects of treatment (placebo- or blocker-implanted) and day (3 or 10) after implantation on vocal behaviours. To control for repeated measures the ID of each territory owner was included as random intercept. Significant results are highlighted in bold.

	during STI		**after STI**	
song duration				
treatment	$F_{\mathbf{1,16}} = 0.07$	p = 0.8	$F_{1,18} = 2.8$	p = 0.1
day of STI	$F_{\mathbf{1,12}} = 0.2$	p = 0.7	$F_{1,17} = 0.3$	p = 0.6
treatment*day	$F_{\mathbf{1,12}} = 8.4$	**p = 0.01**		
duration pause A-B				
treatment	$F_{\mathbf{1,16}} = 0.4$	p = 0.6	$F_{1,18} = 1.5$	p = 0.2
day of STI	$F_{1,13} = 5.2$	**p = 0.04**	$\mathbf{F_{1,13} = 5.8}$	**p = 0.03**
treatment*day	$F_{1,13} = 3.8$	*p = 0.07*		
elements in A				
duration A	$\mathbf{F_{1,12}=17.8}$	**p = 0.001**	$F_{1,12} = 0.2$	p = 0.7
treatment	$F_{1,16} = 0.1$	p = 0.8	$F_{1,18} = 0.2$	p = 0.7
day of STI	$F_{1,12} = 4.2$	*p = 0.06*	$\mathbf{F_{1,12} = 6.7}$	**p = 0.02**
duration A*treatment	$F_{1,12} = 1.6$	p = 0.2	$F_{1,12} = 4.0$	*p = 0.07*
duration A*day			$F_{1,12}= 0.4$	p = 0.5
treatment*day			$F_{1,12} = 0.4$	p = 0.5
duration A*treatment*day			$\boldsymbol{F_{1,12} = 4.7}$	***p = 0.05***
frequency bandwidth A				
duration A	$F_{1,12} = 2.5$	p = 0.1	$F_{1,16} = 0.09$	p = 0.8
treatment	$F_{1,16} = 1.0$	p = 0.3	$\mathbf{F_{1,18} = 5.2}$	**p = 0.04**
day of STI	$F_{1,12} = 0.004$	p = 1.0	$\boldsymbol{F_{1,16} = 4.4}$	***p = 0.05***
duration A*treatment	$F_{1,12} = 3.4$	p = 0.09		
frequency bandwidth B				
duration B	$F_{1,6} = 0.07$	p = 0.8	$\mathbf{F_{1,15}} = 3.1$	0.1
treatment	$F_{1,15} = 0.02$	p = 0.9	$\mathbf{F_{1,18} = 6.2}$	**p = 0.02**
day of STI	$\mathbf{F_{1,6} = 13.7}$	**p = 0.01**	$\boldsymbol{F_{1,15} = 4.2}$	*p = 0.06*
duration B*treatment	$\mathbf{F_{1,6} = 56.1}$	**p = 0.0003**		
duration B*day	$\mathbf{F_{1,6} = 23.5}$	**p = 0.003**		
treatment*day	$\mathbf{F_{1,6} = 31.3}$	**p = 0.001**		
duration B*treatment*day	$\boldsymbol{F_{1,6} = 4.6}$	*p = 0.07*		

elements in C				
duration C	**$F_{1,11}$ = 13.7**	**p = 0.004**	**$F_{1,15}$ = 8.1**	**0.01**
treatment	$F_{1,16}$ = 0.3	p = 0.6	$F_{1,18}$ = 0.005	p = 1.0
day of STI	$F_{1,11}$ = 1.2	p = 0.3	**$F_{1,15}$ = 6.3**	**p = 0.02**
frequency bandwidth C				
duration C	$F_{1,11}$ = 1.3	p = 0.3	$F_{1,15}$ = 0.3	p = 0.6
treatment	$F_{1,16}$ = 0.5	p = 0.5	$F_{1,18}$ = 0.07	p = 0.8
day of STI	$F_{1,11}$ = 0.01	p = 1.0	$F_{1,15}$ = 0.8	p = 0.4

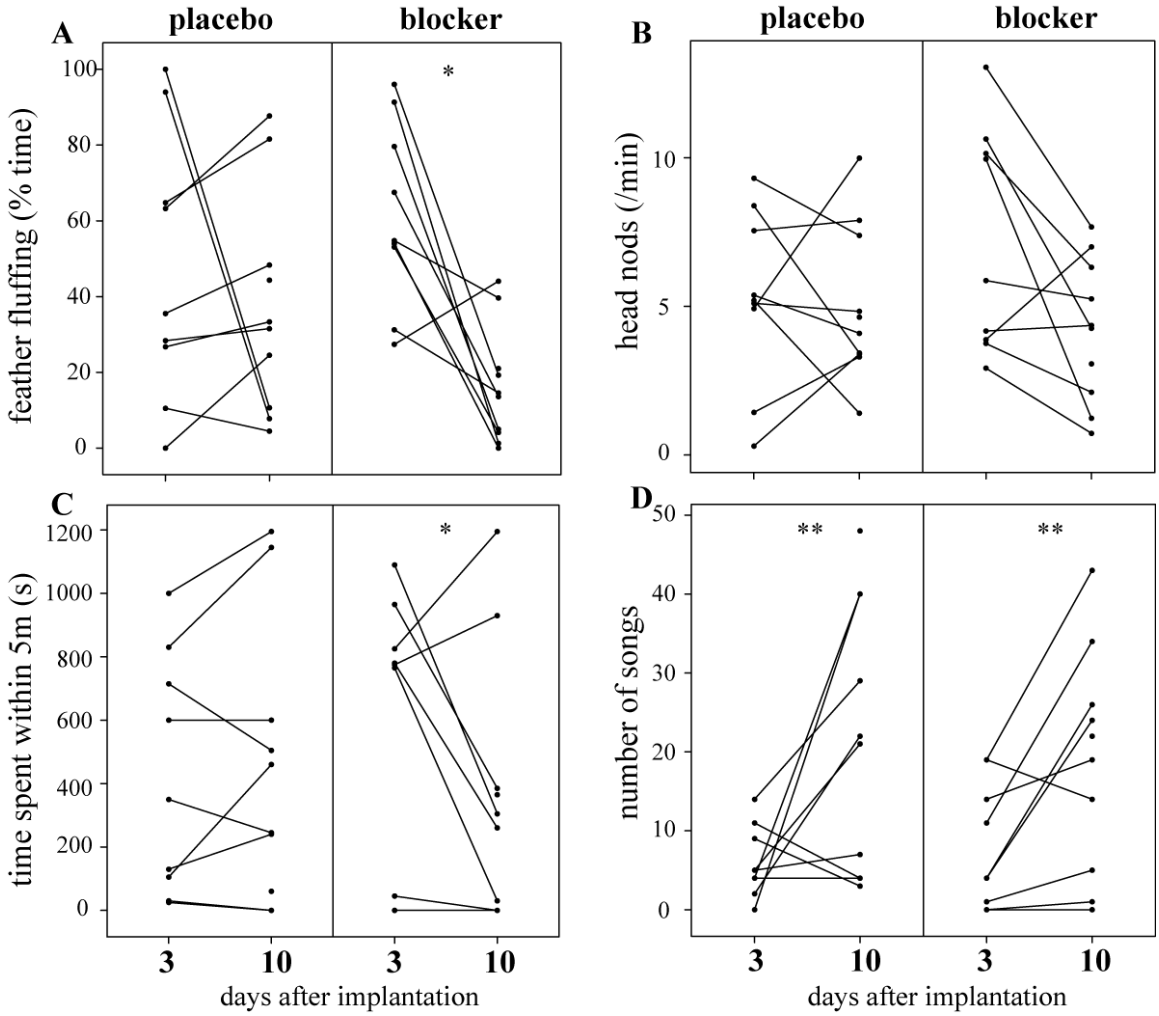

Figure 2: Non-vocal territorial behaviours (A-C) and number of songs (D) in response to simulated territorial intrusions at day 3 and 10 after implantation with an androgen receptor blocker and an aromatase inhibitor. Results for individual males are connected by lines. Asterisks indicate differences between days, * $p < 0.05$; ** $p < 0.01$, see text for details.

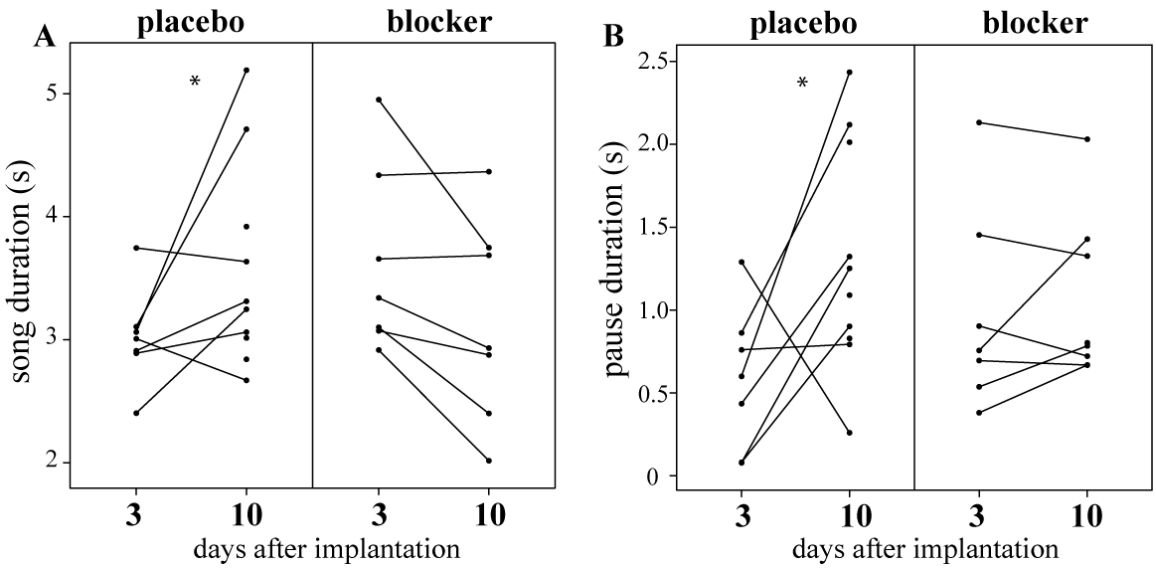

Figure 3: The duration of songs (A) and the duration of the pause between part A and B (B) in response to the STI, separated for males treated with placebo (left) and flutamide and letrozole (right). Each circle represents one individual male. Measurements of the same male are connected by a line. Placebo-implanted males sang significantly longer songs on day 10 than on day 3. Both groups tended to sing with a longer pause duration between part A and B on day 10 than on day 3 after implantation. For details on song measurements and statistics see text (* $p < 0.05$).

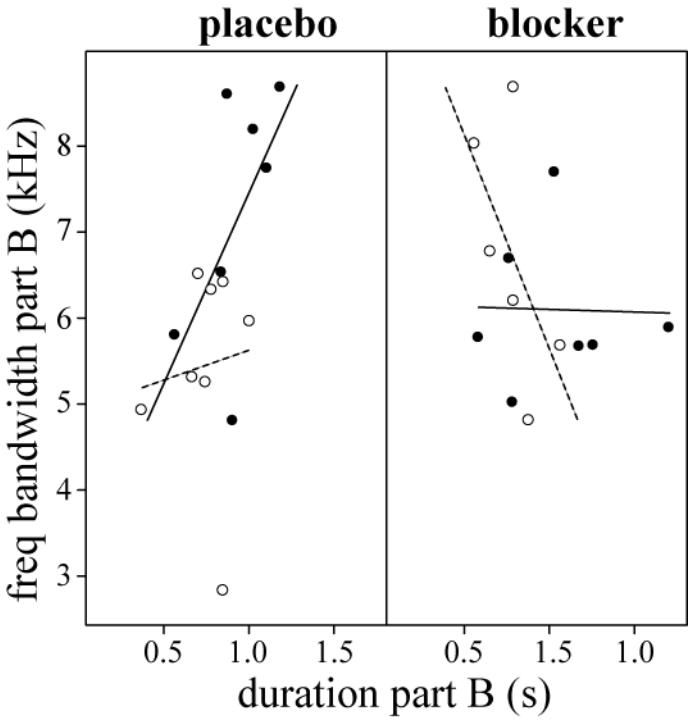

Figure 4: The frequency bandwidth (kHz) of part B plotted against the duration of part B of songs sang in response to the STI, separated for placebo- (left) and blocker-treated males (right; filled circles and solid lines: STI on day 3; open circles and dashed lines: STI on day 10). Lines indicate regression lines.

Behaviour after STI

After the decoy was removed and the playback stopped, placebo- and blocker-implanted males did not differ in the amount of time they spent within 10 m of the decoy on day 3 and day 10 (treatment: $F_{1,18} = 0.5$; day: $F_{1,18} = 1.7$, $p = 0.2$; interaction: $F_{1,18} = 0.2$, $p = 0.7$) and in the number of head nods (treatment: $F_{1,18} = 0.2$, $p = 0.7$; day: $F_{1,17} = 0.1$, $p = 0.7$; interaction: $F_{1,17} = 0.1$, $p = 0.8$).

Also, the number of songs after the STI did not differ significantly between the STIs on day 3 and on day 10 (treatment: $F_{1,18} = 0.004$, $p = 1.0$; day: $F_{1,18} =$

3.1, p = 0.1; interaction: $F_{1,18} = 0.4$, p = 0.5). When controlling for the duration of part A, placebo-implanted males sang more elements in this part on day 10 than on day 3 (Table 3, Fig. 5). Also, blocker-implanted males tended to repeat more elements in part A on day 10 than on day 3, but they did so with a shorter duration of part A compared to control males (Table 3, Fig. 5). Males of both treatment groups sang part C with more elements on day 10 than on day 3 (Table 3). Blocker-implanted males sang part A and B after both STIs with a smaller frequency bandwidth than placebo-implanted males (Table 3, Fig. 6). Thus, while territorial behaviours (except song rate) during the STI were reduced on day 10 compared to day 3, the territorial response after the STI was stronger on day 10 than on day 3 (Table 4): males of both treatment groups sang with a higher vocal performance after the STI on day 10 than on day 3 (more elements in part A and C). This confirms previous findings that the response to STIs is modulated by experience (Apfelbeck, Stegherr et al. 2011, chapter 3). However, the vocal performance of blocker-implanted males after the STI was overall significantly lower than that of placebo-implanted males (smaller frequency bandwidth of part A and B).

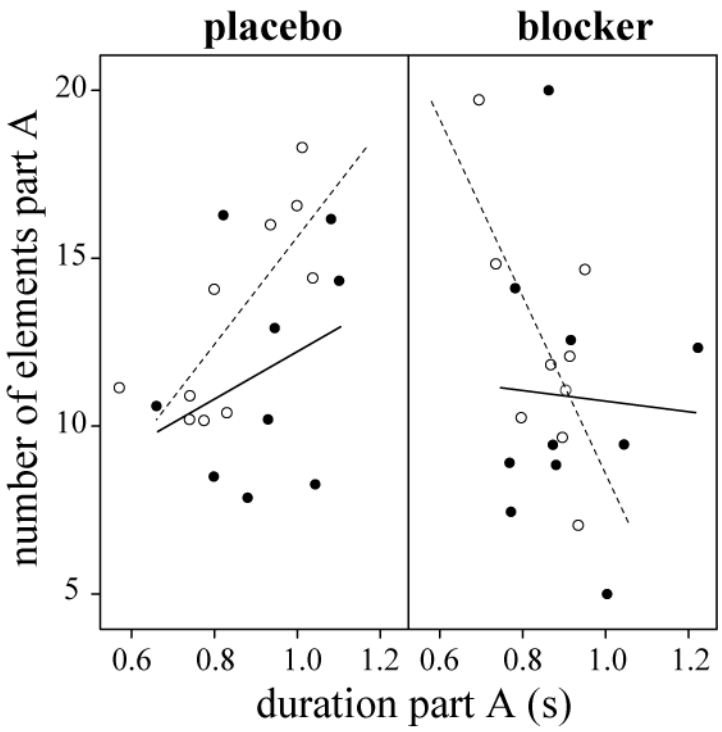

Figure 5: The number of elements in part A plotted against the duration of the part A of songs sang after the STI, separated for placebo- (left) and blocker treated males (right; filled circles and solid lines: STI on day 3; open circles and dashed lines: STI on day 10). Lines indicate regression lines.

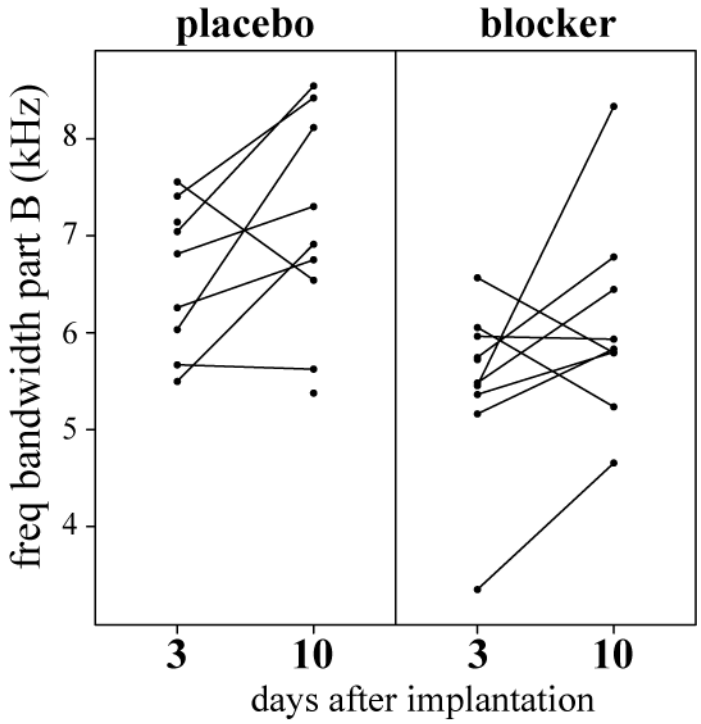

Figure 6: Frequency bandwidth (kHz) of song part B sang after the STI depending on day after implantation, separated for males treated with placebo (left) and flutamide and letrozole (right). Each circle represents one individual male. Measurements of the same male are connected by a line. Placebo-implanted males sang with a significantly broader frequency bandwidth than blocker-implanted males. Both groups tended to sing with a broader frequency bandwidth on day 10 than on day 3 after implantation. For details on song measurements and statistics see text.

Table 4. Relative change in behaviour from day 3 to day 10 in response to the STI and just after the STI. Downward arrows (↓) indicate a quantitative decrease of the behaviour from day 3 to day 10; upward arrows (↑) indicate a quantitative increase of the behaviour from day 3 to day 10. Shaded areas indicate if a response represents a relatively stronger or a weaker territorial response with dark grey shading indicating a stronger response on day 3 than on day 10, and light grey shading indicating a stronger response on day 10 than on day 3. The emerging pattern suggests that both treatment groups reduced their territorial response during the STI from day 3 to day 10, but that this change mainly concerned non-vocal behaviours in blocker-implanted males, but vocal behaviours in placebo-implanted males.

		during STI		after STI	
		placebo	**blocker**	**placebo**	**blocker**
non-vocal response	time spent within 5m		↓		
	feather fluffing		↓		
	head nods		**(↓)**		
vocal response	number of songs	↑	↑		
	song duration	↑		↓	↓
	freq bandwidth B (duration part B)	↓			
	elements in A	↓	↓	↑	↑
	elements in C			↑	↑

Discussion

Inhibiting the effects of testosterone and estrogen did not prevent male black redstarts from successfully defending a territory. However, blocking androgen

and estrogen action changed the emphasis of the territorial response: placebo-implanted males invested more into song related behaviours, while blocker-implanted males invested more into direct approach and non-vocal threat behaviours (Table 4). Furthermore, especially after the STI, vocal performance was generally lower in blocker- than in placebo-implanted males. Thus, black redstarts reacted similar to most other songbird species, in which blocker treatment did not reduce overall territorial behaviours (Table 1). Furthermore, these and results from our previous studies (see below) strengthen the hypothesis that in some species in which territorial behaviour is not restricted to the breeding season, testosterone and oestradiol facilitate only specific components of territorial behaviour that are important in the breeding context. In male black redstarts testosterone and oestradiol seem to emphasize vocalizations relative to non-vocal behaviours in the territorial response. Although males sing spontaneously at a high rate also in autumn, they are less likely to respond to a simulated territorial intrusion with song in autumn than in spring (Apfelbeck et al., in prep., chapter 1, Apfelbeck and Goymann 2011, chapter 2). In contrast, non-vocal territorial behaviours were not reduced in autumn compared to spring (Apfelbeck and Goymann 2011, chapters 1 and 2). Furthermore, male black redstarts increased their vocal performance in response to simulated territorial intrusions compared to spontaneous song before the challenge (Apfelbeck et al., submitted, chapter 4). This change in song structure seems to depend partly on testosterone and/or oestradiol, because both males that were implanted with an anti-androgen and an aromatase inhibitor and males

that were challenged in autumn when testosterone levels were naturally low, did not show the full structural change of their song (Apfelbeck et al., submitted, chapters 4 and 6).

In combination, these data suggest that in male black redstarts testosterone and/or estrogen shift the focus of the territorial response to vocal behaviours and especially facilitate structural changes in the song within an agonistic context.

Evidence from other species support the view that testosterone influences the emphasis of the territorial response. For example, male red-winged blackbirds *(Agelaius phoeniceus)* implanted with an androgen receptor blocker and an aromatase inhibitor spent more time on their territories engaging in aggressive interactions compared to control males, but still lost parts of their territories (Table 1, Beletsky et al., 1990). Also, male European robins *(Erithacus rubecula)* approached intruders more conspicuously during the breeding season than during the non-breeding season, even though the quantitative response (latency to approach, time spent close to the intruder) did not differ between seasons (Schwabl and Kriner, 1991).

Further, in most studies, the quantitative effects of blockers on territorial behaviour were surprisingly weak. Similar to our study on black redstarts, blockers typically reduced only some components of the territorial response (Table 1, but see Soma, Sullivan et al. 1999). These testosterone - regulated components of the territorial response may be especially relevant in a reproductive context, e.g. by acting as a signal to females. Song during territorial

contests in the breeding season, for example, may not only be directed towards the intruding or neighbouring males, but may also convey information to the own mate or other females (e.g. Otter, McGregor et al. 1999). Females pay attention to the performance of their mates during territorial challenges, which may influence female behaviour, i.e. whether they engage in extra-pair copulations or not (Otter, McGregor et al. 1999). Male European starlings only increase their song output in the presence of a female during the breeding season, when testosterone levels are elevated, but not during the non-breeding season (Riters, Eens et al. 2000). Also, male canaries sing more trilled syllables, which are attractive to females, during the breeding season than during non-breeding (Leitner, Voigt et al. 2001). Thus, testosterone or its metabolites may especially activate features of the territorial behaviour that are relevant for females. These features may differ between species (e.g. song rate or song structure), may signal male quality and may be correlated with variation in testosterone levels (Galeotti, Saino et al. 1997). Furthermore, our study demonstrates how important it is to measure the full set of vocal and non-vocal territorial behaviours quantitatively and qualitatively (e.g. song output and song structure). Otherwise subtle effects of the treatment may be missed.

Whether testosterone facilitates the whole suite of territorial behaviours or induces only sexually relevant components of these behaviours in a breeding context, may explain why some songbird species show an increase in androgen levels after simulated territorial intrusions, whereas others do not (reviewed in Goymann, Landys et al. 2007; Goymann 2009). Short-term increases in

testosterone during male-male interactions are thought to enhance the persistence of a territorial response (Wingfield 1994) and may induce the winner effect (Oyegbile and Marler 2005; Oliveira, Silva et al. 2009). These short-term increases in testosterone are a phenomenon found across all vertebrate classes (Wingfield, Hegner et al. 1990; Hirschenhauser, Winkler et al. 2003; Archer 2006; Hirschenhauser and Oliveira 2006), but in many species of birds, these increases in testosterone do not occur (Goymann, Landys et al. 2007; Goymann 2009). Bird species, in which territorial behaviour as such is decoupled from the control of testosterone (e.g. the black redstart), may also lack the effect of short-term increases in testosterone on future territorial interactions. We have previously shown that male black redstarts do not increase testosterone during territorial encounters with other males (Apfelbeck and Goymann 2011, chapter 2). But obviously this does not prevent them from enhancing their territorial response during future territorial encounters with other males (Apfelbeck, Stegherr et al. 2011, chapter 3). Song sparrows, in contrast, increase testosterone during simulated territorial intrusions (Wingfield and Wada 1989) and this increase seems to enhance the persistence of the territorial response after the stimulus is withdrawn (Wingfield 1994). Furthermore, inhibition of aromatase reduces the whole suite of territorial behaviours in response to an intruder in song sparrows, even though the effect is less obvious during the breeding than during the non-breeding season (Soma, Sullivan et al. 2000). Why species differ in the hormonal control of seasonal and short-term territorial aggression is, however, still unclear

(Wingfield and Hunt 2002; Goymann, Landys et al. 2007; Lynn 2008; Goymann 2009; Apfelbeck and Goymann 2011).

Conclusions

In some species – such as black redstarts – that defend a territory during the breeding season (when testosterone levels are high) and also outside the breeding season (when testosterone levels are low), territorial behaviour as such may be decoupled from testosterone or its metabolites. Rather, testosterone or oestradiol may change some components of the territorial repertoire that are specifically relevant in a breeding context. Testosterone may shift the emphasis of the territorial response to these components. Thereby, these behaviours may change signal value and may in turn indicate male quality to other males (intruders and neighbours), but also to females witnessing the territorial dispute. Such context dependent changes in song structure during the breeding season may be facilitated by seasonal changes in testosterone levels. Furthermore, there seem to be fundamental differences between species to which degree territorial behaviour is regulated by testosterone or its metabolites on a seasonal basis. These differences may be directly related to variation in androgen responsiveness to male-male interactions.

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Chapter Six

Autumn territoriality and testosterone in a temperate zone songbird, the black redstart *(Phoenicurus ochruros)*

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Abstract

Seasonal correlations between high levels of testosterone and high frequencies of territorial behaviour and associated song suggest that androgens or its metabolites activate theses behaviours during the breeding season. However, males of several vertebrate species also defend territories during nonbreeding times when testes are regressed and circulating testosterone levels are low. The degree to which sex steroids facilitate territorial behaviours in these species is still not well understood. We studied territorial behaviour in male black redstarts, a temperate-zone songbird, in autumn. Males were implanted both with an antiandrogen and an aromatase inhibitor to block direct and indirect effects of testosterone or with a placebo. In both groups spontaneous song and territorial behaviours including song in response to a simulated territorial intrusion were recorded. Both groups changed structural parameters of their song in the agonistic context, but did not differ in their territorial response. In combination with a previous study in this species in spring, our data show that territorial behaviour per se is independent of androgens and its metabolites in male black

redstarts, but that context and season dependent changes in song structure are facilitated by sex steroids.

Introduction

In many vertebrate species territorial behaviour during the breeding season is correlated with elevated levels of testosterone (Wingfield, Hegner et al. 1990). Furthermore, studies elevating or decreasing testosterone levels using testosterone implants, castration or pharmacological blockers suggest that there may be a causal link between territorial aggression and testosterone at least in some species (Schwabl and Kriner 1991; Canoine and Gwinner 2002; Van Duyse, Pinxten et al. 2002; Weiss and Moore 2004; Sperry, Wacker et al. 2010). However, several species defend territories also outside the breeding season when testes are regressed and circulating testosterone levels are low (Schwabl and Kriner 1991; Wingfield 1994; Canoine and Gwinner 2002; Wingfield and Soma 2002; Scotti, Belén et al. 2008). Studies investigating the role of testosterone and its metabolite oestradiol in territorial aggression during the nonbreeding season are still rare and results are conflicting. Most studies indicate that during that time of the year territorial behaviour may be decoupled from the action of sex steroids (Schwabl and Kriner 1991; Canoine and Gwinner 2002; Scotti, Belén et al. 2008). However, studies on song sparrows *(Melospiza melodia)* found that oestradiol locally produced in relevant brain areas is probably involved in nonbreeding aggression in this species (Soma, Sullivan et al. 1999; Soma, Sullivan et al. 2000; Soma, Tramontin et al. 2000; Soma, Schlinger et al.

2003). Furthermore, sex steroids may be produced de novo in the brain independent of circulating levels (Kazuyoshi and Takeshi 1995; London, Monks et al. 2006).

Song is an important component of territorial behaviour in songbirds and expressed in that context during the breeding and the nonbreeding season in many species (Logan and Hyatt 1991; Wingfield 1994; Weggler 2000). In those species that sing and express territorial behaviour also outside the breeding season there seems to be no difference in song rate between seasons (Wingfield 1994; Van Hout, Eens et al. 2009), although song bout length may be longer in spring than in autumn (Van Hout, Eens et al. 2009). Furthermore, it has been shown in one species that males respond differently to spring and autumn song (Logan and Fulk 1984) indicating that spring and autumn song differ in structure. Spring song may be more stereotyped (Smith, Brenowitz et al. 1997) and males may sing syllables with a higher repetition rate (Logan and Fulk 1984; Smith, Brenowitz et al. 1997; Leitner, Voigt et al. 2001). Trills of fast frequency modulated syllables have been shown to be preferred by females in some species (Vallet, Kreutzer et al. 1997; Ballentine, Hyman et al. 2004) and thus may be more relevant in a reproductive context. Furthermore, breeding season song has been shown to change in an agonistic context in several species (Price, Earnshaw et al. 2006; DuBois, Nowicki et al. 2009). However, despite general comparisons between breeding and nonbreeding song, we are not aware of any study investigating if song changes with context in the nonbreeding season.

Changes in song rate and/or structure from the breeding to the nonbreeding season have been related to a decrease in HVC size (Smith, Brenowitz et al. 1997). HVC is part of the forebrain motor control path that innervates the syrinx and nuclei regulating respiration during song and is androgen sensitive (Gahr and Metzdorf 1997; Bernard, Bentley et al. 1999). It is thought that its size plays a role in the control of complex song. However, not in all species that sing also during the nonbreeding season HVC is smaller during that time of the year than during breeding. In addition, for some species some studies found seasonal differences in HVC size (Nottebohm 1981; Nottebohm, Nottebohm et al. 1986), but others did not (Fusani, Van't Hof et al. 2000). These differences maybe due to distinct delineation methods and seasonal changes seem to be more because of anatomical than of cytochemical changes (Gahr 1997). However, in some species changes in androgen receptor expression in HVC and thus sensitivity to testosterone have been reported (Gahr and Metzdorf 1997; Fraley, Steiner et al. 2010). Still, the role of testosterone in the control of song produced across seasons is unclear.

In this experiment we studied territorial behaviour including song output and structure and its relation to testosterone and its metabolites in male black redstarts during autumn. Previous studies in black redstarts have shown that males increase their vocal performance during a simulated territorial intrusion (STI) in spring, but to a lesser degree when implanted with an antiandrogen and an aromatase inhibitor (Apfelbeck et al., submitted). Furthermore, territory owners implanted with these blockers did not decrease their territorial response

as a whole but shifted the focus of their territorial response: they invested more into direct approach behaviours towards a simulated territorial intruder than placebo-implanted males, while the latter invest more into vocal performance (Apfelbeck et al., in preparation). As a high vocal performance may be an especially relevant signal for females during reproduction and as male black redstarts have very low testosterone levels during autumn (Apfelbeck and Goymann 2011) we hypothesize that territorial behaviour shown by male black redstarts in autumn in a non-reproductive context is independent of testosterone or its metabolites. To test this idea, we compared the territorial behaviour between males implanted with an antiandrogen and an aromatase inhibitor simultaneously and males implanted with a placebo pellet. We also compared song structure and output of song sang spontaneously and song produced in response to a territorial challenge. We predicted that blocker- and placebo implanted males would not differ in their response to a simulated territorial intrusion and that changes in song structure and output should not differ between the two groups.

Methods

Adult (≥ 2 years) male black redstarts were caught in 2009 between September 22 and October 7 in Upper Bavaria (N 47º, E 11º, 500-600m above sea level) with mealworm-baited ground traps by luring them to the site of the traps with short presentation of playback. We remotely muted the loudspeaker as soon as the territory owners approached the traps. Upon capture we took several

biometric measurements and implanted males alternately with either one placebo pellet (n = 8) or two time release pellets (n = 8, Innovative research of America, Sarasota, FL) containing the androgen receptor blocker flutamide and the aromatase inhibitor letrozole respectively (21 day release: 1.5 mg per pellet: 71 µg/day; Ø = 3.2mm). Flutamide blocks the androgen receptor and letrozole inhibits cytochrome p450 aromatase (CYP 19). This enzyme converts testosterone to estrogen (Cheshenko, Pakdel et al. 2008). Thus, by combining flutamide and letrozole direct and indirect effects of androgens were inhibited. Implants were inserted with tweezers under the skin into a small incision on the back between the wings. The incision was sealed with tissue glue (Nexaband; World Precision Instruments).

Control and experimental groups did not differ significantly in body mass ($W = 34.5$, $p = 0.8$), length of the right tarsus ($W = 44$, $p = 0.2$) and length of the right wing (autumn: $W = 41$, p-value = 0.4). Each male was banded with a numbered aluminium ring (Vogelwarte Radolfzell) and a unique combination of three colour rings for individual recognition. Measuring and implanting the birds took no longer than 25 min after which the males were released onto their territories.

To assess if the blocker treatment had any effect on plasma testosterone levels we caught another set of males as described above, but took a blood sample immediately upon capture and brought them to the laboratory. In autumn 2010 (from September 20 to 27) eight males were either implanted with flutamide and letrozole (n = 4) or flutamide alone (n = 4) and bled again three and ten days

after implantation. Males were held in individual cages under natural light conditions.

Playback stimuli

Songs used as playbacks were recorded in autumn 2009 with a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660 (sampling frequency: 44.1 kHz; resolution: 16 bit) from 12 different males. Playbacks were created using Avisoft Saslab pro software (Raimund Specht, Berlin, Germany). Each playback consisted of 20 songs recorded from one male that were filtered (1 kHz high-pass filter) and normalized to the same amplitude (75 %). A playback consisted of each of two different song types played back in a XXYYXXYYXX sequence, with X and Y in 10 different versions. Songs were divided by pauses of 4.5 s. By repeating each sequence six times the playback had a duration of 20 minutes in total. This design resembled the natural song organisation of the species. Eight playbacks were used during one STI only, four playbacks were used twice (once for placebo and once for blocker males).

Simulated territorial intrusion

To asses the effect of the blocker treatment on territorial behaviour we performed simulated territorial intrusion (STI) experiments by placing a stuffed decoy into the centre of the respective territories and playing back black redstart song. STIs were conducted three days after implantation. As decoys we used three different stuffed males in full adult plumage that were protected by an inconspicuous cage made of a wire frame and a mist net and mounted on a tripod. A string attached

to the wire frame allowed to remotely remove the decoy by pulling the string from a distance of about 30 m into a plastic cylinder below the wire frame. Song was played back from a remote-controlled loudspeaker (Foxpro Scorpion, digital game caller, FOXPRO Inc. Lewistown, USA) underneath the decoy at a sound pressure level of 65 dB SPL at 1 m (as measured with a CEL 573.B1 Sound Level Analyser). We recorded the following behaviours of the territory owner for 20 min: (1) latency to respond to the STI either by singing or approaching the decoy, (2) the first time the male was within 5m of the decoy, (3) the time the male spent within 5 m of the decoy, (4) the time the territory owner had its feathers fluffed and (5) the number of head noddings, which are typical threat postures of male black redstarts (Landmann 1996). Furthermore, we noted whether the male attacked the decoy and recorded any song produced. After 20 min the playback was remotely muted and the decoy removed and the behaviour of the territory owner observed and its song recorded for another 10 min. Usually we could determine the location of the male during the whole STI, however, sometimes it was hidden from view and we could not correctly record head nodding and fluffing behaviour. Therefore, we also noted when we knew the location of the bird but could not see it.

Song analysis

Song was analysed using Avisoft-SASLab Pro software, version 4.51. Recordings were visualized in spectrograms (settings: sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50 %). We determined the number of songs by visual inspection and selected songs of sufficient quality (low

background noise) for further sound analysis. Each song of black redstarts can be divided into three distinct parts (part A, B and C; e.g. Cucco and Malacarne 1999) with a pause of varying length between part A and B. We measured the duration of part A, B, C, the total song and the duration of pauses between A and B (see Apfelbeck et al., submitted, chapter 4). We counted the number of elements of part A and C (mean of max. 20 songs). We also determined the frequency bandwidth and the maximum frequency of part A, B and C using the automatic parameter measurement function (threshold -20dB) in Avisoft (mean of max. 10 renditions of high-quality songs).

Plasma separation and hormone analysis

Plasma was separated and stored in 500 µl ethanol at -80°C. Testosterone concentration was determined by direct radioimmunoassay (RIA, following (Goymann, Geue et al. 2006; Apfelbeck and Goymann 2011, chapter 2). Mean ± SD efficiency of the extraction with dichloromethane was 85 ± 5%. Samples were measured in duplicates. The lower limit of detection of the assay was determined as the first value outside the 95 % confidence intervals for the zero standard (B_{max}) and was 0.45 pg/tube. The intra-assay coefficient of variation was 2.9 %. As the testosterone antibody shows significant cross-reactions with 5a-dihydrotestosterone (44 %) our measurements may include a minor fraction of this additional androgen.

Statistical analysis

Treatment groups (placebo, blocker) were compared for differences in their response to a simulated territorial intrusion by using Mann-Whitney U tests for independent non-parametric samples. We analysed the behaviour during and

after the STI separately. After the STI we compared the time spent within 10 m around the decoy instead of 5 m as most males left the immediate surroundings of the decoy and went to higher singing posts. We also analyzed only the number of head nods after the STI as most males almost immediately stopped feather fluffing after the decoy was removed.

Measures of song output and song structure (Table 2) were analysed with linear mixed models for the effects of treatment (placebo, blocker), part of the STI (before STI: spontaneously sung songs; during STI: playback and decoy present; and after STI: directly after removal of playback and decoy) and their interaction. To control for repeated measures the ID of each territory owner was included as random intercept. If the interaction had $\alpha \geq 0.1$ it was removed.

In all cases dependent variables were transformed if assumptions of normality and/or equality of variances were not met. Significance was accepted at $\alpha \leq 0.05$.

Results

Effect of blocker treatment on plasma testosterone levels

Similar to other studies (Schwabl and Kriner 1991; Soma, Tramontin et al. 2000) implantation of flutamide / letrozole combined (n = 4) or flutamide pellets alone (n = 4) had no effect on plasma testosterone levels during the nonbreeding season (Friedman chi-squared = 0.25, df = 2, p = 0.9, Table 1).

Table 1. Plasma testosterone levels before and three and ten days after implantation of either flutamide alone or flutamide and letrozole combined. Numbers in brackets give the range of testosterone levels.

testosterone (pg/ml)	**before**	**3 days after**	**10 days after**
flutamide / letrozole	61.3 (38.7 – 76.3) n = 4	42.8 (25.3 – 54.2) n = 4	35.7 (28.6 – 50.8) n = 4
flutamide	41.8 (29.5 – 64.5) n = 4	47.4 (37.2 – 57.6) n = 4	78.5 (28.8 – 196.4) n = 4

Effect of blocker treatment on territorial behaviour

Non-vocal behaviour during STI

Overall, there was no strong effect of the treatment on territorial behaviour. All implanted males retained their territories. Blocker implanted males nodded significantly less often with their heads than placebo implanted males ($U = 55$, $p = 0.02$, Fig. 1). All other variables did not significantly differ between blocker- and placebo-implanted males, i.e. they did not differ in the latency to approach the decoy ($U = 26$, $p = 0.6$, Fig. 1), in the amount of time they spent within 5m of the decoy ($U = 48$, $p = 0.1$, Fig. 1), in the amount of time with feathers fluffed ($U = 34$, $p = 0.9$, Fig. 1) or the number of flights over the decoy ($U = 40$, $p = 0.4$).

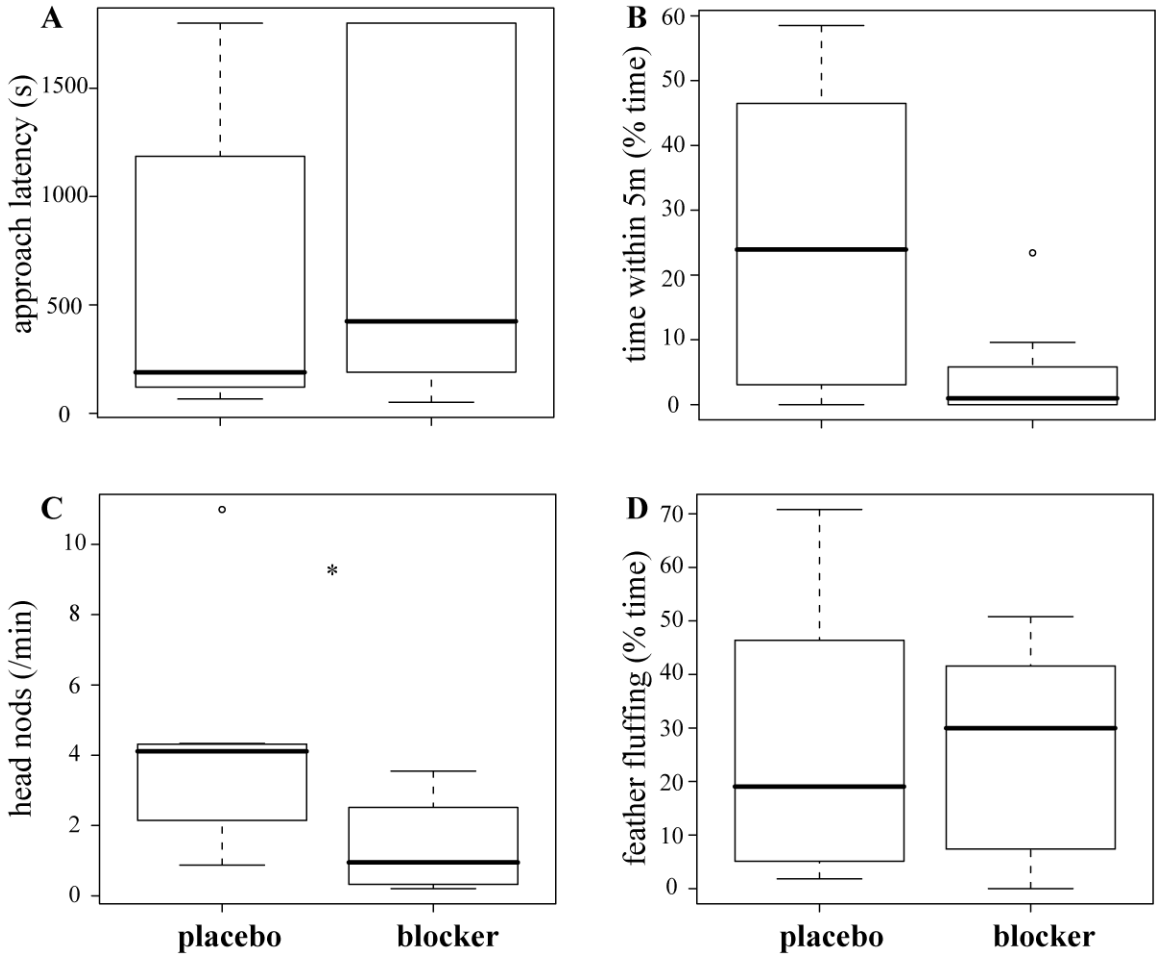

Figure 1: Territorial response to a simulated territorial intruder three days after implantation with a placebo pellet (control, n = 8) or a flutamide and a letrozole pellet (blocker, n = 8). Time within 5 m (B) and time spent feather fluffing (D) are presented as percentage of the time the focal male was seen. Boxplots represent medians and interquartile range. Asterisks indicate statistical significance (* $p \leq 0.05$).

Non-vocal behaviour after STI

Placebo and blocker implanted males did not differ in the amount of time they spent within 10m of the decoy ($W = 47$, $p = 0.1$) and in the number of head nods ($W = 37$, $p = 0.6$) after the decoy was removed and the playback stopped.

Comparison of song output and structure between contexts and treatments

In both treatment groups focal males sang fewer songs during and after the STI than before the experimental challenge (Table 2). There was a non-significant trend that placebo-implanted males sang more than blocker-implanted males (Table 2). Males of *both* treatment groups increased the number of elements in part A and C in response to the experimental challenge (Table 2, Fig. 2) while decreasing the maximum frequency of part A and decreasing the frequency bandwidth of part C (Table 2). Furthermore, males of *both* treatment groups sang part B with a higher maximum frequency in response to the simulated intruder than during spontaneous song (Table 2). However, this latter effect concerning part B should be treated with caution especially in case of the blocker-implanted males (very low sample sizes because of missing values). Thus, similar to spring (Apfelbeck et al., submitted, chapter 4) song changed in the agonistic context, but in contrast to the breeding season these changes were not affected by the blocker treatment.

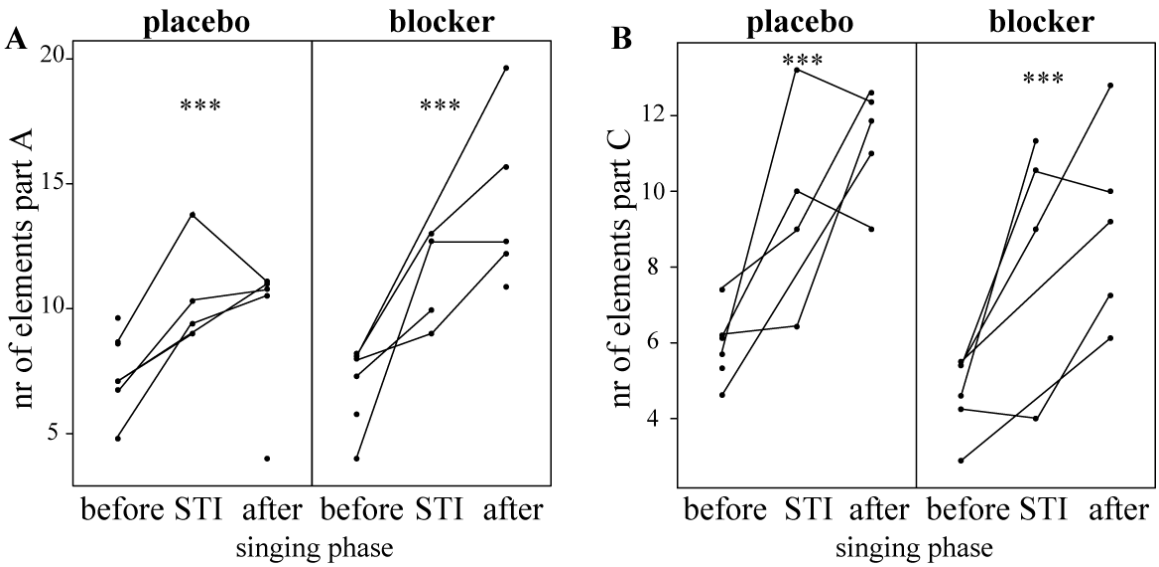

Figure 2: Number of elements in part A (A) and part C (B) before, during and after the STI, separated for males treated with flutamide and letrozole and placebo treated males. Each circle represents one individual male. Measurements of the same male are connected by a line. Asterisks indicate significance (*** $p<0.001$). For details on song measurements and statistics see text.

Table 2. Linear mixed model results for the effects of context and blocker treatment on song output and structure. Part is a within-subjects factor with three levels: before STI (=spontaneously produced songs), during STI (playback and decoy present) and after STI (directly after removal of playback and decoy). Treatment is a between-subjects factor with two levels: placebo-implanted versus blocker-implanted males. Significant results are highlighted in bold.

	treatment	**part**	**part*treatment**
song rate	$F_{1,10} = 3.0$	**$F_{2,18} = 43.1$**	
	p = 0.1	**p < 0.0001**	
song duration	$F_{1,10} = 0.02$	$F_{2,18} = 2.1$	
	p = 0.9	p = 0.1	
duration A	*$F_{1,10} = 4.3$*	*$F_{2,18} = 3.2$*	
	p = 0.06	*p = 0.06*	
duration B	$F_{1,10} = 0.01$	$F_{2,18} = 0.7$	
	p = 0.9	p = 0.5	
duration C	$F_{1,10} = 2.2$	*$F_{2,18} = 3.4$*	
	p = 0.2	*p = 0.06*	
duration pause A - B	$F_{1,10} = 0.4$	$F_{2,18} = 0.1$	
	p = 0.5	p = 0.9	
nr of elements in A	$F_{1,10} = 2.4$	**$F_{2,14} = 11.9$**	*$F_{2,14} = 3.8$*
	p = 0.2	**p < 0.001**	*p = 0.05*
nr of elements in C	$F_{1,10} = 0.2$	**$F_{2,16} = 26.1$**	
	p = 0.2	**p < 0.0001**	
freq bandwidth A	$F_{1,10} = 0.2$	$F_{2,16} = 2.0$	
	p = 0.7	p = 0.2	
max frequency A	$F_{1,10} = 0.08$	**$F_{2,16} = 3.9$**	
	p = 0.8	**p = 0.04**	
freq bandwidth B	$F_{1,10} = 0.7$	*$F_{2,13} = 3.2$*	
	p = 0.4	*p = 0.08*	
max frequency B	$F_{1,10} = 1.5$	**$F_{2,13} = 5.0$**	
	p = 0.2	**p = 0.02**	
freq bandwidth C	$F_{1,10} = 0.03$	*$F_{2,16} = 3.6$*	
	p = 0.9	*p = 0.05*	
max frequency C	$F_{1,10} = 0.1$	$F_{2,16} = 2.2$	
	p = 0.7	p = 0.1	

Discussion

Despite a weak effect on non-vocal behavioural measures there was no effect of the blocker treatment on territorial behaviour in autumn. As predicted, placebo- and blocker implanted males did not significantly differ in the way they changed their song in response to an experimental intrusion. Both treatment groups behaved similarly to blocker-implanted males in spring: they increased the number of elements in part A, but decreased its maximum frequency. In addition, in contrast to placebo-implanted males in spring, they did not shorten the pause between part A and B (Apfelbeck et al., submitted, chapter 4).

These findings strengthen our previous hypothesis that territorial behaviour per se may be decoupled from testosterone in species – such as the black redstart – that defend territories also outside the breeding season, when plasma levels of testosterone are low (Apfelbeck et al., in prep., chapter 5). During spring testosterone may activate those aspects of territorial behaviour that are relevant in a reproductive context and especially important for females. In mockingbirds *(Mimus polyglottos)*, for example, testosterone implants during autumn did not enhance territorial aggression, but activated behaviours associated with mate acquisition: testosterone implanted males sang more, had a higher probability of acquiring a mate and showed more nest building behaviour than control males (Logan and Carlin 1991). Also in European robins *(Erithacus rubecula)* antiandrogen treatment had only a weak effect on territorial behaviour during the breeding season and no effect at all in autumn. Although in the latter study song structure was not measured, Schwabl and Kriner also

suggested that testosterone may stimulate the incorporation of sexy song features during spring (Schwabl and Kriner 1991). In wild and domesticated canaries *(Serinus canaria)* males sing a higher number of fast frequency-modulated syllables during the breeding season (Leitner, Voigt et al. 2001; Voigt and Leitner 2008). Female canaries prefer these fast frequency-modulated syllables (Draganoiu, Nagle et al. 2002). Thus, this trait may be under the control of testosterone and/or oestradiol. Similarly, when comparing spontaneous song across seasons in male black redstarts, males sing more elements in part A and C of their song during breeding than in autumn (Apfelbeck et al., in prep., chapter 1). However, the increase in the number of elements in these parts in response to a simulated territorial intrusion was independent of the treatment with blockers both in spring (Apfelbeck et al., submitted, chapter 4) as well as in autumn (present study) and occurred in both seasons. The effect of the treatment in spring was most pronounced for structural frequency components of the song (Apfelbeck et al., submitted, chapter 4). Furthermore, the changes in song in response to simulated territorial intrusions in autumn were not significantly different between treatment groups and were more similar to blocker-implanted males in spring than to placebo-implanted males during the same period. This indicates that, at least in male black redstarts, the maintenance of a high maximum frequency is facilitated by testosterone, but a high repetition rate of elements in part A and C is not facilitated by this hormone. Recent studies show that maximizing syllable repetition rate and frequency bandwidth simultaneously may be an indicator of male quality and is assessed by females (Ballentine,

Hyman et al. 2004; Ballentine 2009). Thus, although the number of repetitions per song part change seasonally (Apfelbeck et al., in prep., chapter 1), only context-dependent changes in song structure seem to be androgen or oestrogen dependent.

These changes in song may be regulated by aromatase activity in the pre-optic area (Riters and Ball 1999; Riters, Eens et al. 2000) or changes in androgen and oestrogen sensitivity in the song control nucleus HVC (Gahr and Metzdorf 1997) as we found a higher expression of aromatase mRNA in the pre-optic area in spring than in autumn, but no seasonal change in HVC size in another study on male black redstarts (Apfelbeck et al., in prep., chapter 1).

Conclusions

Already in 1988, Moore proposed a model for the regulation of territorial behaviour across different seasons with changing levels of testosterone (Moore 1988). Based on a comprehensive series of experiments in male mountain spiny lizards *(Sceloporus jarrovi)* he suggested that territorial behaviour in such study systems may rely on sex steroid dependent and independent pathways which may either act additive or synergistically. Our studies on male black redstarts and a literature review on blocker and castrations studies in songbirds (Apfelbeck et al., in prep., chapter 5) support this view. In the case of black redstarts the effects seem rather additive than synergistically.

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General discussion

Our studies have shown that in a temperate-zone songbird, the black redstart, the link between testosterone and territorial behaviour is much weaker than is often assumed in birds and other highly seasonal vertebrates. First, based on several experiments it seems very unlikely that territorial interactions modulate androgen levels in males (chapters 2 and 3). Second, seasonal comparisons and blocking direct and indirect effects of testosterone imply that in male black redstarts testosterone does not facilitate territorial behaviour as a whole, but only influences some components (chapters 1, 4, 5, 6).

Male-male interactions do not modulate androgen levels in male black redstarts (chapters 2 and 3)

Natural plasma androgen levels in male temperate zone vertebrates vary markedly between individuals even when sampled during the same breeding stage. This is especially well documented for songbirds and has gained considerable attention recently (Ball and Balthazart 2008; Kempenaers, Peters et al. 2008; Williams 2008). Social interactions between males are thought to be one major factor influencing short and long-term fluctuations in androgen levels within individuals in most vertebrates, including humans (Wingfield, Hegner et al. 1990; Hirschenhauser, Winkler et al. 2003; Hirschenhauser, Taborsky et al. 2004; Archer 2006; Hirschenhauser and Oliveira 2006). Although in several species interactions between males lead to short-term increases in androgen levels, comparative studies in songbirds have shown that this is actually the case

in only about half of the species tested to date (Goymann, Landys et al. 2007; Goymann 2009). The number of broods or the length of the breeding season have been suggested to be environmental factors that may explain between species differences in androgen responsiveness to male-male interactions (Wingfield and Hunt 2002; Goymann, Landys et al. 2007; Landys, Goymann et al. 2007). The comparative studies and the number of broods / short breeding season hypotheses are based on data from distinct species. We tested the number of broods / short breeding season hypotheses for the first time in populations of the same species breeding at different altitudes and therefore differing in the length of their breeding season (chapter 2). We, therefore, could test these hypotheses independent of possible confounding phylogenetic effects. Male black redstarts breeding at high altitudes should not increase testosterone in response to simulated territorial intrusions. As they have only a very short breeding season and often no time for a replacement clutch, the success of this one brood is crucial for the reproductive success of a pair. High levels of testosterone have been shown to interfere with paternal care (Silverin 1980; Hegner and Wingfield 1987) and therefore may jeopardize the success of the brood. In contrast, male black redstarts breeding at lower altitudes should increase testosterone in response to simulated territorial intrusions. Their breeding season is long enough to allow for further and replacement clutches. Thus, the boost in territorial aggression because of a surge in testosterone should outweigh its potential costs. Our studies in male black redstarts show, however, that this hypothesis does not explain the absence of an androgen

response in black redstarts. Although high-altitude and low-altitude male black redstarts reacted aggressively to simulated territorial intrusions, males of both populations did not increase testosterone in response to this behavioural challenge (Apfelbeck and Goymann 2011, chapter 2). However, despite the lack of a direct androgen response to simulated territorial intrusions, black redstarts of both populations would have had the physiological capacity to raise testosterone: males that did not show an increase in testosterone during simulated territorial intrusions showed a pronounced increase in testosterone when injected with gonadotropin releasing hormone (GnRH; Apfelbeck and Goymann 2011). GnRH is released by the hypothalamus and via several steps stimulates the testes to produce testosterone (Nelson 2005). So far it was unknown, if species that show no androgen response to aggressive interactions have already maximally elevated testosterone levels. Our data demonstrate for the first time that at least in black redstarts this is not the case; they are not physiologically constrained to increase testosterone during social challenges. Furthermore, the data show that the length of the breeding season or the number of broods cannot universally explain between-species differences in androgen responsiveness to territorial challenges as males from the high-altitude and the low-altitude population responded to simulated territorial intrusions and GnRH in a similar way (chapter 2).

The function of short-term increases in testosterone in other species is still barely understood. Testosterone may enhance persistence of aggression during or after a fight and may facilitate the winner effect (Wingfield 1994; Oliveira, Silva

et al. 2009; Dijkstra, Schaafsma et al. 2012). California mice (*Peromyscus californicus*), for example, show a short-term increase in testosterone only after experiencing and winning several territorial encounters (Oyegbile and Marler 2005). Studies testing this “winner-challenge”-hypothesis are, however, still rare and have been conducted under laboratory conditions only. As male black redstarts have the physiological capacity to further increase testosterone, we tested if free-living male black redstarts may need several territorial challenges before a significant rise in plasma testosterone would occur (chapter 3). These experiments demonstrate that even repeated simulated territorial intrusions did not result in an increase in androgens and that different intrusion intensities had a strong effect on behaviour but not on hormone levels (Apfelbeck, Stegherr et al. 2011, chapter 3). Furthermore, we could show that a lacking increase in circulating androgen levels after simulated territorial intrusions is probably not due to the induction of a loser effect as had been proposed (Kempenaers, Peters et al. 2008). Because the experiments were conducted in the field, we could not directly test if a winner effect exists in black redstarts. However, our behavioural data strongly suggest that this is the case. Similar to species in which a winner effect was demonstrated, males responded faster to the intruder during following encounters than during the first encounter (Hsu, Earley et al. 2006). Because no increase in testosterone occurred even after multiple intrusions, our data suggest that - although the winner effect seems to be a very general phenomenon among vertebrates (Hsu, Earley et al. 2006) - its regulation may differ between species and may be independent of sex steroids in some species.

The role of testosterone in territorial behaviour expressed across different seasons (chapters 1, 4, 5 and 6)

It is thought that territorial behaviour of temperate-zone vertebrates is regulated by testosterone, because seasonal testosterone profiles closely match the occurrence of territorial behaviour in males (Lincoln, Guinness et al. 1972; Wingfield, Hegner et al. 1990; Klukowski and Nelson 1998). However, several songbird species, including black redstarts, also maintain territories in the non-breeding season when testosterone levels are low (Schwabl and Kriner 1991; Wingfield 1994; Canoine and Gwinner 2002; Apfelbeck and Goymann 2011; Hau and Beebe 2011; Marasco, Fusani et al. 2011). Male black redstarts had completely regressed testes and very low plasma testosterone levels in autumn and also did not increase testosterone levels in response to GnRH (Apfelbeck and Goymann 2011, chapters 1 and 2). Nevertheless, they defended territories as vigorously in autumn as during the breeding season: they responded to a simulated territorial intrusion with a high intensity and sang spontaneously at a high song rate (Apfelbeck and Goymann 2011, chapters 1 and 2). However, the likelihood to respond to simulated territorial intrusions with song was significantly lower and when singing spontaneously they sang fewer elements in the repetitive parts of their song during non-breeding than during breeding (chapter 1). Correlational evidence that testosterone facilitates territorial behaviour in male black redstarts is, thus, inconclusive: non-vocal territorial behaviours seem to be independent from testosterone action, but vocal territorial behaviours may be regulated by testosterone. Furthermore, in both seasons neural centres

associated with song production and brain regions known to facilitate sexual and agonistic behaviours (pre-optic (POA) and hypothalamic areas) express androgen and oestrogen receptor mRNA as well as aromatase mRNA and thus potential direct and indirect binding sites for testosterone (Gahr and Metzdorf 1997; Metzdorf, Gahr et al. 1999; Gahr 2001, chapter 1). Furthermore, experiments in another species suggest that - despite low levels of circulating testosterone - sex steroids still may regulate territorial behaviour in the non-breeding season by local conversion of androgens into oestradiol directly in the brain (Soma, Tramontin et al. 2000) and using substrates from nongonadal origin (Soma and Wingfield 2001). We, therefore, implanted male black redstarts in the breeding season and in autumn with an androgen receptor blocker and an aromatase inhibitor to block both possible direct and indirect effects of testosterone on territorial behaviour (chapters 4, 5, 6). Control groups were implanted with placebo pellets. Inhibiting the effects of testosterone and oestrogen simultaneously did not prevent male black redstarts from successfully defending a territory. In both seasons, males did not lose their territories and the overall response of focal males to simulated territorial intrusions was not significantly lower in blocker- than in placebo-implanted males (although there was a weak trend in autumn; chapters 5 and 6). However, while the blocker treatment had no strong overall effect on territorial behaviour, blocking androgen and oestrogen action changed the emphasis of the territorial response during the breeding season: placebo-implanted males invested more into song related behaviours, while blocker-implanted males invested more into direct approach

and non-vocal threat behaviours (chapter 5). Furthermore, especially after the simulated territorial intrusion vocal performance was in general lower in blocker- than in placebo-implanted males. In addition, we found that male black redstarts increased their vocal performance in response to simulated territorial intrusions: they sang parts of their song with a higher number of elements and a broader frequency bandwidth compared to spontaneous song just before the simulated territorial intrusion (chapters 4 and 6). This change in song structure seems to be partly testosterone and/or oestrogen dependent, because both males that were implanted with an anti-androgen and an aromatase inhibitor simultaneously and males that were challenged in autumn when testosterone levels were naturally low, did not show the full structural change of their song (Apfelbeck et al., submitted, chapters 4 and 6). The change in emphasis of the territorial response and/or the change in song structure in the agonistic context may be facilitated by the conversion of testosterone to oestradiol in the pre-optic area as male black redstarts expressed more aromatase mRNA in that area at the beginning of the breeding season than in autumn (chapter 1). For example, in European starlings the increase in song rate in response to females and other courtship behaviours correlate with a high activity of aromatase in this brain region (Riters, Eens et al. 2000; Pintér, Péczely et al. 2011). Also, seasonal changes in androgen sensitivity in the song control nucleus HVC, but not variation in HVC size, may facilitate changes in song structure as treatment with an androgen receptor blocker inhibited these structural changes in song in spring and black redstarts in autumn behaved similar to blocker-implanted males in spring. However,

testosterone probably does not regulate seasonal changes in element number in parts of the spontaneous song (chapter 1). Also other species show seasonal changes in the production of repetitive elements (Smith, Brenowitz et al. 1997; Leitner, Voigt et al. 2001; Voigt and Leitner 2008). It has been suggested that these changes depend on changes in HVC size and androgen sensitivity of HVC (Smith, Brenowitz et al. 1997). Our data show, that at least in black redstarts, this is not the case because HVC was not significantly smaller during non-breeding than during breeding and administration of an androgen receptor blocker had no effect on the number of elements produced.

In conclusion, these results support the hypothesis that in some species, in which territorial behaviour is not restricted to the breeding season, testosterone and oestradiol facilitate only some components of the territorial behaviour in a breeding context. Our correlational and experimental data suggest that in male black redstarts testosterone and oestradiol emphasize vocalizations relative to non-vocal behaviours in the territorial response. Furthermore, testosterone specifically facilitates structural changes in the song in an agonistic context during the breeding season (chapters 4 and 6). Even though other studies have indicated and suggested that testosterone may only facilitate some components of the territorial response in species that express territorial behaviour across seasons (Schwabl and Kriner 1991; Hau, Wikelski et al. 2000; Van Duyse, Pinxten et al. 2002; Sperry, Wacker et al. 2010), our studies are the first to provide substantial evidence based on a series of experiments and seasonal correlations. In addition, even though changes in song in response to

territorial challenges are commonly found in other species as well (Price, Earnshaw et al. 2006; DuBois, Nowicki et al. 2009), our studies are the first to show that these changes may be facilitated by testosterone and its metabolites. Furthermore, our studies also indicate how testosterone and its metabolites may implement their effects on territorial behaviour on a neuronal basis.

The interrelationship between territorial behaviour and testosterone: are there fundamental mechanistic differences between species?

Whether testosterone facilitates the whole suite of territorial behaviours or just sexually relevant components of it during the breeding season, may also explain why some songbird species show an increase in androgen levels after interactions with other males over territories and others do not. Short-term increases in testosterone during male-male interactions are thought to enhance persistence of the territorial response (Wingfield 1994), facilitate the winner effect (Oyegbile and Marler 2005; Oliveira, Silva et al. 2009) and are a phenomenon found across all vertebrate classes (Wingfield, Hegner et al. 1990; Hirschenhauser, Winkler et al. 2003; Archer 2006; Hirschenhauser and Oliveira 2006). However, especially in birds, there are several exceptions (Goymann, Landys et al. 2007; Goymann 2009). Bird species such as black redstarts, in which territorial behaviour as such is decoupled from control by testosterone, also seem to lack short-term androgen modulation during territorial interactions. However, further studies that measure both the androgen responsiveness to male-male interactions and the role of testosterone in regulating territorial

behaviour would be needed to test this idea. Why species differ in the hormonal control of seasonal and short-term territorial aggression is, however, still unclear (Wingfield and Hunt 2002; Goymann, Landys et al. 2007; Lynn 2008; Apfelbeck and Goymann 2011). Apart from the hypotheses that we have tested (chapter 2), it has been suggested that tropical species may regulate territorial behaviour differently from temperate-zone species (Moore, Walker et al. 2004; Hau and Beebe 2011). Yet, northern temperate black redstarts actually seem to be more similar to tropical species than to some other temperate-zone species (Wingfield and Wada 1989; Soma, Tramontin et al. 2000). Furthermore, there may be phylogenetic differences: the relationship between territorial behaviour and testosterone may have evolved several times independently in different genera. However, within the genus *Zonotrichia*, an exceptionally well studied taxon, there seems to be considerable variation within this group of related birds (Wingfield and Wada 1989; Wingfield and Hahn 1994; Soma, Tramontin et al. 2000; Meddle, Romero et al. 2002; Moore, Walker et al. 2004).

Future studies

Our data indicate that in some species that defend territories across seasons with changing circulating testosterone levels only some components may be activated by testosterone during the breeding season. These components of the territorial response may be especially relevant in a reproductive context, e.g. by acting as a signal to females. Song during territorial contests in the breeding season, for example, may not only be directed towards intruding or neighbouring

males, but may also convey information to the own mate or other females (e.g. (Otter, McGregor et al. 1999)). It has been shown previously that repeated ("trilled") syllables with a high frequency bandwidth are challenging to produce (Podos 1997; Podos 2001). Therefore, such syllables may represent an honest signal of male quality (Ballentine 2009) and are a preferred song feature of females in some species (Vallet, Kreutzer et al. 1997; Draganoiu, Nagle et al. 2002; Ballentine, Hyman et al. 2004). Our experiments suggest that testosterone and/or oestradiol may be the underlying mechanism ensuring the honesty of vocal performance as an indicator of male quality and that especially behaviours that are relevant for females in a reproductive context are under control of sex steroids in male black redstarts. Thus, although interactions between males have no influence on male androgen levels, interactions with females might have (Moore 1982; Moore 1983; Gwinner, Van't Hof et al. 2002) and may be one factor explaining the large variation in testosterone levels between males at the beginning of the breeding season. We have started to test this hypothesis by prolonging the receptive phase of female black redstarts by implanting them with oestradiol at the end of the breeding season (Apfelbeck et al., unpublished data). If the fertility status of their mate influences male testosterone levels, males that are paired with oestradiol-implanted females should postpone the drop of testosterone levels that normally occurs during the last brood (see chapter 1). In addition, differences in testosterone levels may reflect male quality by influencing vocal performance during agonistic contexts. If vocal performance during agonistic encounters indeed reflects male quality, vocal performance should be

correlated with measures of male quality (e.g. body mass (see for example Ballentine 2009), immune response, feather quality, etc.). If testosterone translates male quality into vocal performance during an agonistic encounter, also testosterone should be correlated with vocal performance (Galeotti, Saino et al. 1997; Safran, Adelman et al. 2008). Furthermore, future studies on black redstarts should investigate the threat value of their song and the response of females to variation in vocal performance in playback experiments. If females prefer males with a high vocal performance during territorial contexts, these males should have a higher within- and extra-pair mating success. Thus, variation in testosterone levels and vocal performance should also translate into fitness differences. Furthermore, to distinguish if the effects on vocal performance are due to direct androgen action or occur because of conversion to oestrogens, territorial males should be implanted with flutamide and letrozole separately.

Conclusions

In summary, the experiments demonstrate that androgen levels in male black redstarts are not socially modulated and interactions between males over territories cannot account for the high inter-individual variability in plasma testosterone levels between males. However, as the potential for a further increase in testosterone is given during the whole breeding season, other factors, like the presence of receptive females, may influence male androgen levels. In addition, differences in testosterone levels may reflect male quality by influencing

vocal performance during agonistic contexts. Furthermore, the data show that in male black redstarts territorial behaviour as such is probably not facilitated by testosterone and/or its metabolites. Instead sex steroids seem to activate only some components of territorial behaviour during the breeding season and emphasize these components in the territorial response. In black redstarts sex steroids facilitate context-dependent changes in song structure and strengthen vocal behaviour. Maintaining high testosterone levels for a prolonged period of time has been shown to have major costs (Wingfield, Lynn et al. 2001) including early death (Dufty 1989). Thus, species that defend territories for extended periods of time and also independent of reproduction may have lost the direct regulation of territorial behaviour by androgens to avoid the costs of testosterone. Leaving only sexually relevant components of territorial behaviour under the control of sex steroids, but decoupling the rest of the territorial response from it, seems to be one potential mechanism. However, other species may have solved this problem differently (Moore 1988; Soma, Sullivan et al. 2000) highlighting a diversity in physiological mechanisms that is largely unexplored.

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